TROPICAL FOREST ECOSYSTEM RESPONSES TO INCREASING NUTRIENT AVAILABILITY

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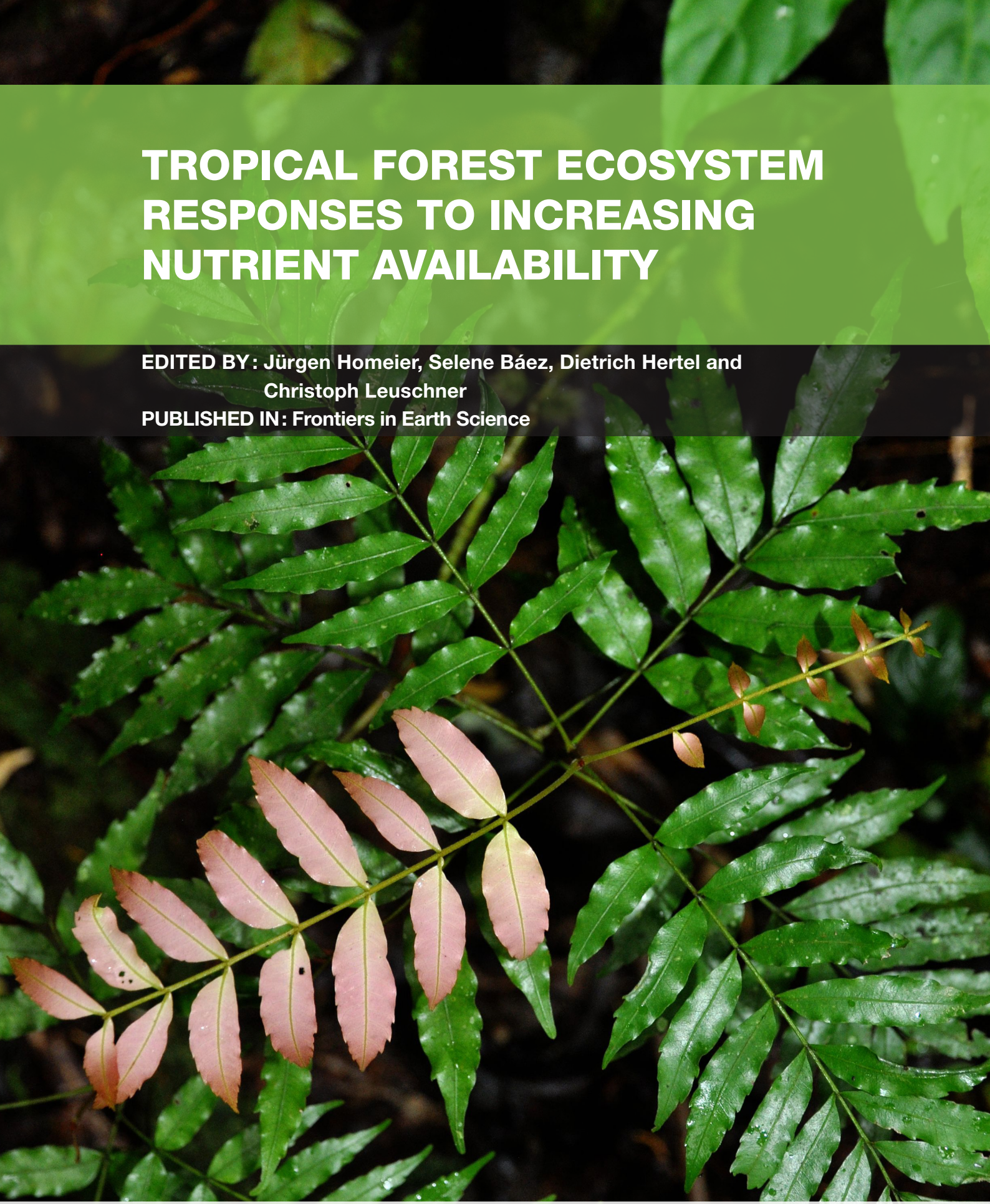

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TROPICAL FOREST ECOSYSTEM RESPONSES TO INCREASING NUTRIENT AVAILABILITY

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Seedling of *Alfaroa costaricensis* (Standl.) J.-F. Leroy (Juglandaceae), a tropical premontane tree species (Photo Jürgen Homeier).

Deforestation and land use change have led to a strong reduction of tropical forest cover during the last decades. Climate change will amplify the pressure to the remaining refuges in the next years. In addition, tropical regions are facing increasing atmospheric inputs of nutrients, which will have unknown consequences for the structure and functioning of these systems, no

matter if they are within protected areas or not. Even remote areas are expected to receive rising amounts of nutrients.

The effects of higher rates of atmospheric nutrient deposition on the biological diversity and ecosystem functioning of tropical ecosystems are poorly understood and our knowledge of nutrient fluxes and nutrient limitation in tropical forest ecosystems is still limited. Yet, it will be of paramount importance to know the effects of increased nutrient availability to conserve these ecosystems with their biological and functional diversity.

During the last years, research efforts have more and more focused on the understanding of the role of nutrients in tropical ecosystems and several coordinated projects have been established that study the effects of experimental nutrient addition.

This Research Topic combines results from experiments and from observational studies with the aim to review and conclude on our current knowledge on the role of additional nutrients in ecosystems.

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EDITORIAL
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Editorial: Tropical Forest Ecosystem Responses to Increasing Nutrient Availability

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Editorial on the Research Topic

Tropical Forest Ecosystem Responses to Increasing Nutrient Availability

Human activity is nowadays altering the structure and functioning of ecosystems in most regions of the world. While deforestation and conversion to agricultural land are the most visible threats to tropical forests worldwide, these systems are also increasingly exposed to atmospheric nutrient deposition and climate change (Lewis et al., 2009, 2015; Malhi et al., 2014; Newbold et al., 2014). Even the most remote tropical forest areas are expected to receive rising amounts of air-borne nutrients, which will have unknown consequences for the biogeochemical cycles, structure and functioning of these systems, no matter if they are within protected areas or not (e.g., Wilcke et al., 2013; Fernández-Martínez et al., 2014). In the decades to come, both climate change and atmospheric deposition likely will amplify the pressure exerted on the remaining tropical forests. Today, effects of global warming are already recognizable in the structure and species composition of tropical forests (e.g., Duque et al., 2015; Báez et al., 2016), and the foliar N concentrations of tropical trees have been found to increase in certain regions (e.g., Hietz et al., 2011). However, the effects of increased atmospheric nutrient deposition on the biological diversity and ecosystem functioning of tropical forests are currently poorly understood. No doubt, politicians, forest managers and scientists need a mechanistic understanding of the processes of change that are triggered by continued nutrient addition, in order to sustainably manage and conserve these systems in the future.

Only in the last decade, increasing research efforts have been directed toward the understanding of the consequences of nutrient inputs for tropical ecosystems, including several replicated field experiments (e.g., Wright et al., 2011; Homeier et al., 2012). This special issue brings together the results from studies exploring the effects of experimental nutrient manipulation in tropical forests at different organization levels, ranging from single species to the ecosystem level. Six of the nine articles in the issue deal with the ongoing interdisciplinary nutrient manipulation experiment in perhumid Andean forests of southern Ecuador (NUMEX). They address a variety of topics, notably effects of N and/or P addition on soil carbon and nitrogen concentrations (Velescu et al.), soil phosphatase activity (Dietrich et al.), and soil N_2O fluxes (Müller et al.) and soil aggregation (Camenzind et al.), and they deal with the response of tree seedlings to increased nutrient availability (Cárate-Tandalla et al.) and changes in the wood anatomy of a locally common tree species (Spannl et al.). The paper by Powers et al. reviews the diversity of nutrient addition effects on tropical dry forests, and Ostertag and DiManno review plant foliar responses to N and P fertilization in different ecosystems based on a literature survey. Hofhansl et al. discuss potential interactive effects of nutrient addition and increasing atmospheric [CO_2] in tropical Amazonian lowland forests as studied in the AmazonFACE and AFEX experiments. All studies report significant changes in the studied ecosystem components or processes in response to nutrient

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addition, highlighting the importance of even small alterations in the nutrient cycle of tropical forests for their functioning.

The magnitude of potential tropical forest responses to increasing nutrient availability depends on the compartment considered and the amount and duration of nutrient addition, ranging from minor alterations in biogeochemical cycles or altered growth rates to shifts in species composition, biotic homogenization and markedly altered ecosystem services. Reactions at all organization levels are likely to be modified or amplified by feedback with other disturbances and could lead to a decrease of ecosystem resilience. Eventually, long-term nutrient deposition may result in alternative stable forest states or even novel ecosystems. Because of the ecological complexity of tropical forests with different forest types and even the species within a given forest responding in different ways to nutrient addition, well-designed comprehensive studies combining manipulative approaches with long-term monitoring are needed to be able to predict the fate of these systems during the next decades.

Future studies should ideally combine the monitoring of climate, element fluxes and forest functioning with an evaluation of species composition and species performance (e.g., Zhou et al., 2013; Fayle et al., 2015; Trumbore et al., 2015; Mori et al., 2017). The recently obtained results suggest that study periods should have a minimum extension of 10 years, since climate variation and specific weather events can interfere with the effects of altered nutrient availability. Since most tropical forests are by far too diverse to study every species, we suggest focusing primarily on abundant taxa and important functional species groups. Finally, to further understand how human activities are affecting tropical forest, modeling approaches are needed that integrate the observed process responses and help understand the mechanistic relationships that drive the change.

AUTHOR CONTRIBUTIONS

JH organized the Research Topic together with SB, DH, and CL.

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ORIGINAL RESEARCH
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Response of Dissolved Carbon and Nitrogen Concentrations to Moderate Nutrient Additions in a Tropical Montane Forest of South Ecuador

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In the past two decades, the tropical montane rain forests in south Ecuador experienced increasing deposition of reactive nitrogen mainly originating from Amazonian forest fires, while Saharan dust inputs episodically increased deposition of base metals. Increasing air temperature and unevenly distributed rainfall have allowed for longer dry spells in a perhumid ecosystem. This might have favored mineralization of dissolved organic matter (DOM) by microorganisms and increased nutrient release from the organic layer. Environmental change is expected to impact the functioning of this ecosystem belonging to the biodiversity hotspots of the Earth. In 2007, we established a nutrient manipulation experiment (NUMEX) to understand the response of the ecosystem to moderately increased nutrient inputs. Since 2008, we have continuously applied 50 kg ha^{-1} a^{-1} of nitrogen (N), 10 kg ha^{-1} a^{-1} of phosphorus (P), 50 kg + 10 kg ha^{-1} a^{-1} of N, and P and 10 kg ha^{-1} a^{-1} of calcium (Ca) in a randomized block design at 2000 m a.s.l. in a natural forest on the Amazonia-exposed slopes of the south Ecuadorian Andes. Nitrogen concentrations in throughfall increased following N+P additions, while separate N amendments only increased nitrate concentrations. Total organic carbon (TOC) and dissolved organic nitrogen (DON) concentrations showed high seasonal variations in litter leachate and decreased significantly in the P and N+P treatments, but not in the N treatment. Thus, P availability plays a key role in the mineralization of DOM. TOC/DON ratios were narrower in throughfall than in litter leachate but their temporal course did not respond to nutrient amendments. Our results revealed an initially fast, positive response of the C and N cycling to nutrient additions which declined with time. TOC and DON cycling only change if N and P supply are improved concurrently, while NO_3-N leaching increases only if N is separately added. This indicates co-limitation of the microorganisms by N and P. The current increasing reactive N deposition will increase N export from the root zone, while it will only accelerate TOC and DON turnover if P availability is simultaneously increased. The Saharan dust-related Ca deposition has no impact on TOC and DON turnover.

Keywords: nutrient manipulation experiment, nitrogen (N) phosphorus (P) and calcium (Ca) additions, total organic carbon (TOC), dissolved organic nitrogen (DON), nitrate leaching, tropical montane forest, Ecuador

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INTRODUCTION

Tropical forests generate one third of the terrestrial gross primary production (Beer et al., 2010), absorb large amounts of carbon dioxide (CO_2) from the atmosphere (Schimel et al., 2014) and store a considerable amount of carbon (C) in the biomass (Jobbagy and Jackson, 2000). Thus, they play a key role in regulating concentrations of greenhouse gases in the atmosphere and global climate. However, they are vulnerable to environmental change and perturbation by human activities. Climate change (Vuille et al., 2003; Urrutia and Vuille, 2009) and nitrogen (N) deposition (Galloway et al., 2004, 2008; Hietz et al., 2011) are current environmental changes which might threaten the functioning of the native tropical ecosystems in the north Andes.

The tropical Andean forest in south Ecuador has experienced improved nutrient supply in the past 15 years because of steadily increasing N and episodic calcium (Ca) and magnesium (Mg) deposition (Boy and Wilcke, 2008; Wilcke et al., 2013a). The observed N deposition is mainly attributable to biomass burning resulting from the conversion of natural forests to agricultural land in the Amazon Basin (Galloway et al., 2004, 2008; Da Rocha et al., 2005; Boy et al., 2008). On the other side, dust particles rich in Ca and Mg originating from the Sahara are transported over the Atlantic and deposited from the atmosphere on the eastern slopes of the Andean cordillera mainly during La Niña events (Kaufman et al., 2005; Boy and Wilcke, 2008; Wilcke et al., 2013b). Phosphorus (P) is predominantly deposited as mineral aerosol dust transported with the trade winds from the Sahara to the Caribbean and the Amazon Basin (Pett-Ridge, 2009). At the same time, increasing air temperature and increasingly unevenly distributed rainfall have allowed for longer dry spells and reduced air humidity (Peters et al., 2013), resulting in a reduction of the water content in the thick organic layer, where the activity of soil organisms is highest.

Dissolved organic matter (DOM) serves as a C and energy source for microorganisms and is an essential component of the global C and N cycles (Van Hees et al., 2005; Kalbitz and Kaiser, 2008; Bolan et al., 2011). Leaching of vegetation canopies, litterfall and soil organic matter are the most important sources of DOM (Kalbitz et al., 2000). There is strong indication that the fate of DOM in soils is controlled by microbial degradation (Kalbitz et al., 2000). Thus, less waterlogging could favor mineralization of DOM by microorganisms and hence increase nutrient release from the organic layer into the mineral soil (Roman et al., 2010).

As a result of further agricultural and industrial development in the Amazon Basin (Walker et al., 2009), biomass burning and thus N supply with easterly winds is expected to continue in the near future. This will result most likely in further changes of the N cycle in the remote natural montane ecosystems of south Ecuador, where dissolved organic nitrogen (DON) dominates the N cycling (Goller et al., 2006; Wilcke et al., 2013a). Increasing N richness can furthermore result in dissolved N losses from terrestrial ecosystems to ground and surface waters, posing a potential threat to water quality (Matson et al., 1999; Corre et al., 2010). Moreover, elevated supply of easily plant-available inorganic N forms—ammonium (NH_4^+) and nitrate (NO_3^-) – might favor only certain plant species by stimulating biomass production in a way that could lead to changes in species composition and ultimately even to a reduction of species richness in the megadiverse tropical montane forest ecosystem of south Ecuador, which belongs to the global biodiversity hotspots of the Earth (Myers et al., 2000; Phoenix et al., 2006).

In the fragile montane ecosystems, it is frequently assumed that nutrient constraints limit plant growth and primary production, which are known to be regulated by the supply of key elements, like N and P (Vitousek and Howarth, 1991; Tanner et al., 1998). In younger soils, N limitations were alleged to be more frequent, since N must be accumulated from the atmosphere, while P limitations often occur in older soils, because availability of P, which is derived from the parent material, is declining during soil formation (Walker and Syers, 1976). However, this model does not take into account that thick organic layers with high nutrient stocks develop in many tropical montane forests, which may provide sufficient N for tree growth (Wilcke et al., 2002) especially within the context of changing environmental conditions (e.g., reduced waterlogging and increasing air temperature).

Since nutrient limitation was thought to be widespread in tropical soils, P would usually limit productivity in lowland rain forests growing on heavily weathered soils, while N, in contrast, would be the limiting nutrient in montane rain forests growing on less weathered young soils (Tanner et al., 1998). Single nutrient limitations which vary with soil age were tested in several studies, where N additions to tropical montane forests and P additions to lowland forests increased forest growth and primary production (Vitousek, 1984; Tanner et al., 1990, 1992; Vitousek et al., 1993; Adamek et al., 2009). Vitousek et al. (2010) investigated relations between N and P limitations and suggested depletion, soil barriers, and low-P parent material as mechanisms controlling the ecosystem mass balance of P.

Recently, single nutrient limitations have begun to be questioned. The study of Kaspari et al. (2008) in a tropical lowland forest in Panama revealed that nutrient limitations may vary not only between, but also within ecosystems, so that different limitations occur in the canopy, in the organic layer or in the mineral soil and that nutrient limitation can also vary temporally. In the same forest, potassium (K), phosphorus (P), or nitrogen (N) were shown to limit root allocation, tree growth, or litter production (Wright et al., 2011). At the same time, extractable nutrients showed a variable response to fertilizer addition and to the seasonality of the climate (Turner et al., 2013). In the tropical montane forest of south Ecuador, incubation experiments suggested that soil microorganisms might also be limited by calcium, manganese, sulfur, and zinc (Wilcke et al., 2002). In forests of the Oregon coast range, Ca cycling was found to be coupled to the N cycle, particularly if N concentrations in the ecosystem ranged between N limitation and N saturation (Perakis et al., 2006). One of the most comprehensive meta-analyses of studies on nutrient additions emphasized that both N and P limitations were widespread in terrestrial systems and revealed synergistic effects of combined N and P enrichment, which prevailed over individual element limitations (Elser et al., 2007). Multiple nutrient co-limitations

were also found in grassland ecosystems for aboveground net primary production (Fay et al., 2015), including N, P, K, and several other micronutrients. In a large-scale study in the Peruvian Andes and Amazonia, Fisher et al. (2013) showed that nutrient–productivity relationships depend on elevation, indicating strong N and P co-limitation at sites up to 1000 m a.s.l. and strong N limitation at sites above 1000 m a.s.l. These results demonstrate that various nutrient co-limitations may occur in different ecosystems and may be widespread across multiple elevation and latitudinal gradients.

However, constraints to primary production may be different from limitations of organic matter cycling. A large amount of nutrients is stored in the organic layer of tropical montane forest soils. Thickness of organic layers was shown to increase with elevation, because of decreasing temperatures and increasing soil humidity, which inhibit mineralization of organic matter and rates of nutrient turnover (Wilcke et al., 2002, 2008; Arnold et al., 2009). Litter decomposition was limited by N and P in a tropical forest in Hawaii, while litterfall and stem growth were only limited by N (Hobbie and Vitousek, 2000). In another nutrient addition experiment in a montane rain forest in Panama, N additions increased not only stem growth and litterfall production (Adamek et al., 2009), but also N-cycling rates in the soil as well as NO_3 leaching, resulting in a more open N cycle (Corre et al., 2010).

Nutrient limitations play an important role in the control of C and N cycling in tropical montane forests. Improved nutrient availability favors microbial activity and an elevated N supply is known to increase the turnover of light, easily degradable organic matter, while it further stabilizes heavier, more recalcitrant compounds of the organic matter pool (Neff et al., 2002; Janssens et al., 2010). Fisher et al. (2013) also reported that addition of N was associated with an increase in microbial respiration, which confirms results revealing increased decomposition rates of easily degradable litter as a result of N additions (Janssens et al., 2010). Bragazza et al. (2006) reported a biologically controlled increase in dissolved organic carbon (DOC) release from peat with increasing N deposition in a laboratory incubation of peat bogs from different European sites with varying N deposition rates. For 17 sites across northwest Europe and North America Evans et al. (2008) did not find a consistent effect of experimental N application on DOM concentrations (in nine cases DOC concentrations increased in response to N application and in eight cases DOC concentrations decreased). Acidifying N application (as NH_4^+) mostly decreased DOC concentrations while non-acidifying N application (as NO_3^- or NH_3) increased DOC concentrations. There was no general relationship between N addition rate or N concentrations in soil solution and DOC concentrations. A positive correlation between C/N ratios of the organic layer with DOC concentrations in soil solutions reported by Borken et al. (2011) for a number of south German forests suggests that increasing N availability decreases DOM concentrations, although this does not seem to be generally true (Michalzik et al., 2001). In a tropical montane forest in the south Ecuadorian Andes, Baldos et al. (2015) found that N additions led to a decrease in K_2SO_4 extractable C, indicating a reduction in the easily degradable C fraction in the organic layer. Finally, at an adjacent study site, results from a long-term monitoring at micro-catchment scale revealed steadily decreasing DON concentrations and fractional contribution of DON to total N in rainfall, throughfall and soil solutions over 15 years (Wilcke et al., 2013a). The observed decreasing organic contributions to total N were not only the result of increasing inorganic N deposition over 15 years, but could also be attributable to enhanced DON decomposition, which might be in turn related with reduced waterlogging occurring simultaneously with higher nutrient availability.

Studies conducted during the last decade often stressed that N emissions to the atmosphere and the increasing use of fertilizers in the modern world strongly interfere with natural nutrient cycling (Galloway et al., 2004, 2008). In the past century, the N cycle was markedly changed in the northern hemisphere because of human-induced N emissions to the environment and the use of mineral fertilizers to increase crop yields (Van Breemen, 2002; Galloway et al., 2004, 2008; Erisman et al., 2008). A widely used method to determine the response of ecosystems to increased nutrient supply and to determine which elements might have limiting effects on ecosystem processes is to set up fertilizer experiments, were nutrients are applied at a high rates to ecosystems (up to 250 kg ha^{-1} a^{-1}), to trigger fast responses that can be observed within short time (Tanner et al., 1998; LeBauer and Treseder, 2008; Fisher et al., 2013). However, these rates are much higher than actual or expected nutrient deposition of a few tens of kg ha^{-1} a^{-1} (Galloway et al., 2004, 2008). Because responses to low level fertilizer amendments might only be visible after longer periods, studies investigating effects of moderate nutrient additions are rare (Gaige et al., 2007; Fang et al., 2009).

In summary, investigations in tropical montane forests have shown that a considerable uncertainty still persists with regard to the potential response of the N and DOM cycling in these ecosystems to increasing supply of nutrients from atmospheric deposition and from climatically favored, enhanced mineralization of soil organic matter. Therefore, our objectives were to elucidate the response of (i) the N cycle in a tropical montane forest and (ii) the dissolved organic C and N concentrations and TOC/DON ratios to nutrient additions. We hypothesized that (i) N and Ca additions increase N concentrations while P additions (alone and in combination with N) reduce N concentrations in throughfall and soil solutions and (ii) that increasing nutrient availability decreases TOC and DON concentrations and widens the TOC/DON ratios. We tested these hypotheses in a nutrient manipulation experiment (NUMEX), in which N, P, N+P, and Ca were fertilized at moderate levels considered as reflecting realistic future changes in nutrient availability.

MATERIALS AND METHODS

Study Area

The study area is located in the province of Zamora-Chinchipe, on the Amazon-exposed slopes of the south Ecuadorian Andes (3.58° S, 79.08 W). The experimental plots are situated in the Reserva Biológica San Francisco at an altitude between 2010 and

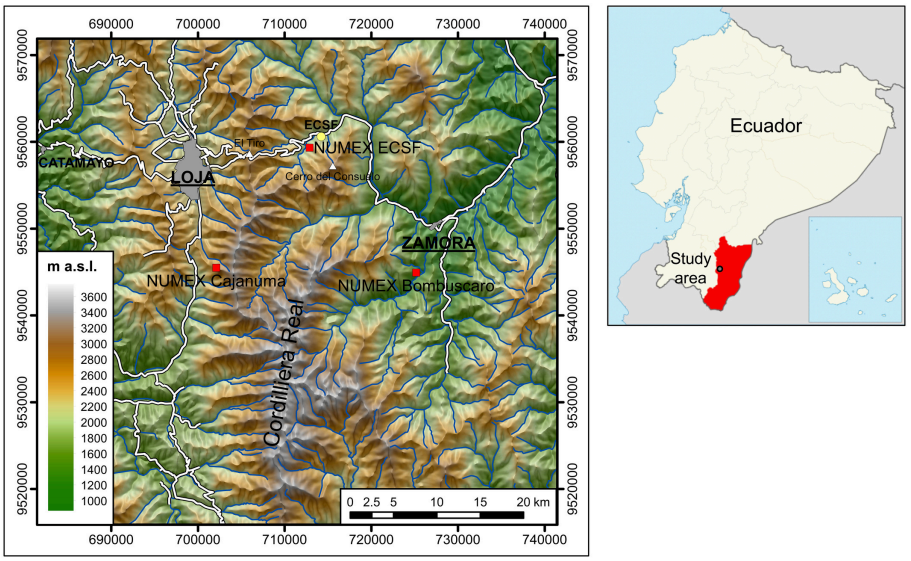

FIGURE 1 | Location of the research area and of the Nutrient Manipulation Experiment (NUMEX). Our study focuses on the experimental site at 2000 m a.s.l. near the Research Station San Francisco (ECSF). Coordinate system: WGS84, UTM Zone 17 M. Cartographic data: Instituto Geográfico Militar (2013).

2128 m a.s.l. (**Figure 1**). The slope of the plots ranges between 15 and 40°, with an average of 28°. Precipitation is unimodally distributed over the year and reaches its maximum between April and July, yet without a marked dry season. Between 1999 and 2014, the annual rainfall averaged 2291 ± 228 mm (SD). During the same period, at the meteorological station at 1957 m a.s., the mean annual temperature was 15.4 ± 0.3°C and the mean air humidity was 83.5 ± 1.4%. The warmest month was November with 16.4 ± 0.6°C and the coldest month was July with 14.5 ± 0.7°C. Temperature and humidity were aggregated from meteorological data of the ECSF climate station (Rollenbeck et al., 2015).

The vegetation cover consists of a near-natural, little disturbed lower montane forest (Bruijnzeel and Hamilton, 2000; Homeier et al., 2008), which has an extremely high species richness and is considered to be a hotspot of biodiversity (Barthlott et al., 2005). The mean canopy height reaches 12–14 m and the mean crown radius ranges between 2 and 4 m, reaching up to 5–6 m. The most abundant families are Lauraceae, Melastomataceae, and Rubiaceae, with *Graffenrieda emarginata* Triana (Melastomaceae) being the most abundant tree species (Homeier and Werner, 2007). The canopy cover is very dense (Leaf Area Index of 5.19 to 9.32, Fleischbein et al., 2005) and retains with an evaporative interception loss of about 47% a high proportion of the incident precipitation (Fleischbein et al., 2006; Wullaert et al., 2009).

The soils of the experimental area are young and mostly shallow (<60 cm), have a loamy texture and were classified according to IUSS Working Group WRB (2006) as Stagnic Cambisols (Hyperdystric, Chromic), which developed from Palaeozoic phyllites, quartzites, and metasandstones belonging to the Chiguinda unit of the Zamora series (Hungerbühler, 1997). The mineral soil is covered by a thick organic layer (30 ± 9 cm) with a very low bulk density (0.08 ± 0.01 g cm^{-3}), which constitutes the main rooting zone for plants and trees (Wilcke et al., 2002).

Experimental Design

NUMEX was established in the year 2007 and is continued until present. It consists of three experimental sites along an altitudinal gradient at 1000, 2000, and 3000 m a.s. (**Figure 1**). Details on all three NUMEX sites are given by Martinson et al. (2010) and Homeier et al. (2012). We focus on the part of the experiment at 2000 m a.s.l. located in the Reserva Biológica San Francisco. NUMEX is an interdisciplinary experiment and consists of low level, continuous additions of N, P, combined N and P, Ca, and unfertilized control plots. Nutrient additions started in January 2008 and take place twice a year. The fertilizers were applied directly to the forest soil (Wullaert et al., 2010). Special care was taken to insure the homogeneous distribution of the fertilizer on the experimental plots.

The experiment site includes 20 plots (20 × 20 m) in a fourfold replicated, randomized block design (**Figure 2**). Since 2008, we have continuously applied 50 kg ha^{-1} a^{-1} of N, 10 kg ha^{-1} a^{-1} of P, 50 kg + 10 kg ha^{-1} a^{-1} of N and P, and 10 kg ha^{-1} a^{-1} of Ca. The fertilization rates were similar to the values of annual natural deposition (**Figure 3**), since our aim was to study the response of the forest ecosystem to a moderate increase of nutrient input. Because of Ecuadorian regulations which impeded the use of ammonium nitrate (NH_4NO_3), N was applied as commercially available urea (46% N). P was applied as sodium di-hydrogen phosphate ($NaH_2PO_4 \cdot H_2O$, AppliChem GmbH, Darmstadt, Germany) and Ca as calcium chloride ($CaCl_2 \cdot 2\ H_2O$, Merck, Darmstadt, Germany). Both used chemicals were of analytical reagent grade.

Field Sampling

In 2007, all study plots were equipped with throughfall collectors, zero-tension litter lysimeters, and suction cups at the 0.15 and 0.3 m mineral soil depths.

Throughfall was collected with 20 rain gauges (Hellmann type) which were randomly distributed on each plot along two perpendicular transects, resulting thus in 400 collectors for the whole experiment area. To ensure sampling representativeness, number and spatial distribution of the throughfall collectors were determined taking into account the spatial and temporal variability of precipitation, which was analyzed in a separate experiment between 2004 and 2008 (Wullaert et al., 2009). The collectors consisted of a 2000-mL wide mouth PE bottle, and a PE funnel with a diameter of 118 mm closed at the lower end with a polyethylene net with a 0.5 mm mesh size, to avoid contamination by litter debris and insects. All bottles were wrapped in aluminum foil to reduce irradiation effects and a table-tennis ball was placed inside the funnels to reduce evaporation. Volumes of collected throughfall were measured in the field with a graduated cylinder and samples were bulked plotwise, resulting in 20 volume-weighted samples per collecting date. To guarantee a homogeneous chemical quality of the sampling throughout the duration of the experiment, throughfall collectors were regularly cleaned with distilled water and temporarily covered with plastic bags during fertilization to avoid direct contamination by the applied chemicals.

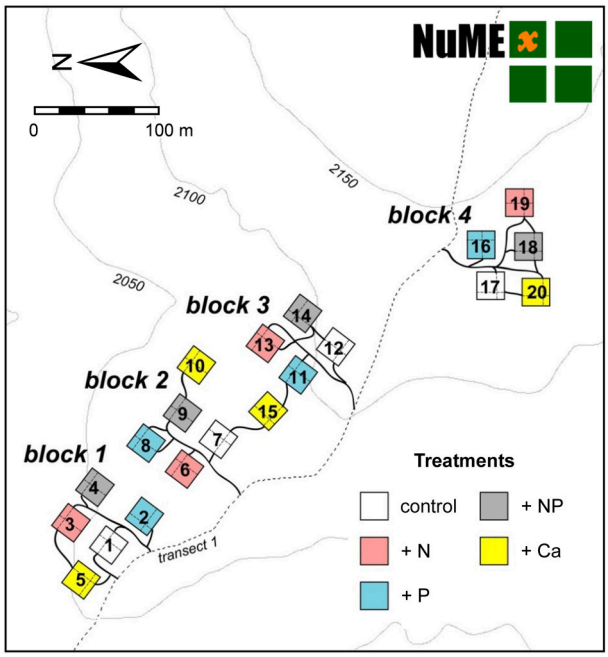

FIGURE 2 | Layout of the Nutrient Manipulation Experiment (NUMEX) in the Reserva Biológica San Francisco at 2000 m a.s.l. The experiment site includes 20 plots in a fourfold replicated, randomized block design (four nutrient addition plots and one control treatment in each block).

Litter leachate (solution percolating through the organic layer) was collected using three zero-tension lysimeters per plot, which consisted of plastic boxes covered with a polyethylene net with a 0.5 mm mesh size, connected to sampling bottles, which were wrapped in aluminum foil. The lysimeters were installed underneath the organic layer at 7–51 cm below ground surface from the side of a small soil pit, at upper, middle and lower slope positions, taking care that the organic layer itself was only minimally disturbed during or after installation. Litter leachate was bulked plotwise after individual sampling of the collecting bottles, resulting thus in 20 samples per collecting date.

Throughfall and litter leachate have been collected fortnightly since August 2007. Incident rainfall was collected weekly at 2–4 gauging stations and five rain collectors at each station, with the same Hellmann-type collectors used for sampling throughfall. After collection, throughfall and litter leachate samples were

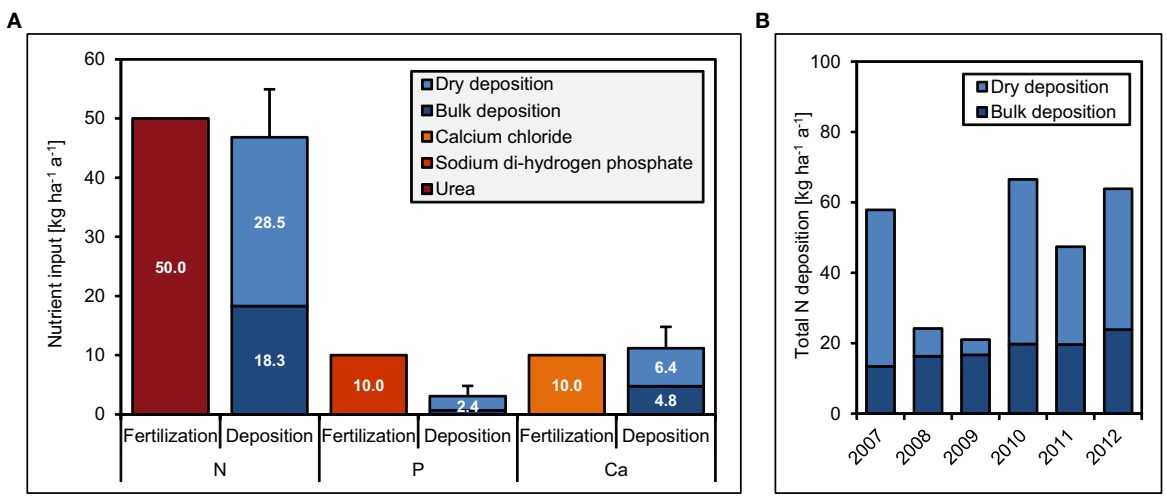

FIGURE 3 | Fertilization and deposition rates between 2007 and 2012 in the Nutrient Manipulation Experiment (NUMEX). The diagrams show **(A)** annual fertilization and deposition rates of N, P, and Ca and **(B)** total annual N deposition during the study period. Error bars indicate one standard error of average total deposition rates over the 5 years.

transferred to the field laboratory of the Estación Científica San Francisco (ECSF), where they were immediately filtered through ashless filter paper, pore size 4–7 μm, Type 392 (Sartorius-Stedim GmbH, Göttingen, Germany) and then kept at −20°C until shipping in frozen state to Switzerland for chemical analysis.

Chemical Analyses and Quality Control

For this study, we analyzed a dataset of 5 complete years including a fortnightly record of element concentrations in throughfall and litter leachate between 2007 and 2012, which we mainly measured in the laboratory of the Geographic Institute at the University of Bern, Switzerland. Electrical conductivity (TetraCon 325, WTW GmbH, Weilheim, Germany) and pH values (Sentix 81, WTW GmbH, Weilheim, Germany) were immediately measured in unfiltered aliquots of the collected water samples in the laboratory of the ECSF in Ecuador.

Concentrations of the dissolved N species ammonium-N (NH_4-N), nitrate-N (NO_3-N) and total nitrogen (TN) were measured by continuous flow analysis (CFA) using high resolution colorimetry and photometric detection (AutoAnalyzer 3 HR, Seal GmbH, Norderstedt, Germany). During the CFA measurements, samples were dialyzed and TN was oxidized to nitrate by UV digestion with potassium peroxydisulfate and sodium hydroxide in presence of a boric acid buffer. NH_4-N was determined by the Berthelot reaction and NO_3-N by cadmium reduction. The determination of NO_3-N concentrations by cadmium reduction usually includes a small amount of nitrite-N (NO_2-N), which was below the detection limits and hence considered negligible. We measured TOC (filtered through 4–7 μm pores) as non-purgeable organic carbon (NPOC) by elemental analysis through high temperature combustion and infrared (IR) detection (varioTOC cube, elementar Analysensysteme GmbH, Hanau, Germany), after acidifying 10 mL of the sample with 50 μL of a 10% HCl solution to remove inorganic C.

Average detection limits were 0.024 mg L^{-1} for NH_4-N, 0.016 mg L^{-1} for NO_3-N, 0.042 mg L^{-1} for TN and 0.16 mg L^{-1} for TOC. The quality of the CFA measurements was verified by running certified N standards (Merck, Darmstadt, Germany) with a concentration of 0.4 mg L^{-1} NH_4-N and 0.5 mg L^{-1} NO_3-N in each measured batch. Certified standards were combined with in-house control standards, with a concentration of 0.5 mg L^{-1} NH_4-N and 0.25 mg L^{-1} NO_3-N, which were run on average every 20 samples. The quality of TOC measurements was verified by use of in-house control standard only, which had a concentration of 5 mg L^{-1} and 10 mg L^{-1} TOC. Measurement batches were only accepted if the results for the control standards deviated by less than 10% from the target values. The relative SD of the measurement precision of the control standards was ± 4% for NH_4-N, ± 2% for NO_3-N, ± 5% for TN, and ± 3% for TOC.

Calculations and Statistical Evaluation

Throughfall fluxes were measured fortnightly in the field, averaged per plot and summed up to monthly and annual data. Concentrations of dissolved organic nitrogen (DON) were calculated as difference between TN and the sum of NH_4-N and NO_3-N concentrations. Values below the detection limit were taken as zero for calculations, possibly resulting in an underestimation of the DON concentrations. Monthly mean concentrations were calculated by averaging fortnightly concentrations of each measured element. Annual mean element concentrations were calculated as volume-weighted means of monthly concentrations. The effects of nutrient addition on the investigated element concentrations were expressed as log response ratios (RRx), which were calculated as a natural logarithm of the ratios between the measured values in the nutrient addition treatments and in the control plots. A value of 0.2 indicates thus a relative difference in concentrations of 22% between treatment and control, while a value of 0.5 indicates a relative difference of 65%.

We performed statistical analyses with the software R, version 3.0.2 (R Core Team, 2013). To test for pre-existing differences among the 20 plots of the experiment with respect to element concentrations in throughfall and litter leachate, we performed an analysis of variance (ANOVA) on data collected before the first fertilization (August 2007 to December 2007). To reveal differences in element concentrations in throughfall and litter leachate between treatments and control plots and compare different effects among treatments after the start of nutrient additions in 2008, we used linear mixed effects models implemented in the R-package *lme4* (Bates et al., 2015) to perform a repeated measures analysis. We tested for homogeneity of variances and insured normal distribution of the residuals by visual inspection. We specified treatments as fixed effects, with the four blocks as random factor over the 5 years of the experiment, and calculated significance levels of the fixed effects with the functions *cftest* and *glht* in the package *multcomp* (Hothorn et al., 2008).

Time series of element concentrations in monthly data were analyzed using the seasonal Mann-Kendall test, as suggested by Hirsch et al. (1982), which is included in the R-package *Kendall* (McLeod, 2011). This non-parametric test estimates trends in time series while correcting for seasonality (Helsel and Hirsch, 2002). The rank correlation coefficient tau (τ) measures the strength of the temporal trend. Since it is a sign test, it is robust against outliers and can be used in spite of data gaps, which commonly occur when there is not enough sample volume collected in the field. Linear regression lines in our figures are only meant to illustrate detected significant trends and do not necessarily imply that these trends are linear.

RESULTS

Water Fluxes

We did not detect significant differences between treatments and control plots either before the start of nutrient additions or during the 5 years of the experiment (**Table 1**). We collected throughfall quantitatively in high spatial resolution, because it is the most relevant water flux which also controls the amount of water percolating through the organic layer. Consequently, in the following we consider

TABLE 1 | Throughfall fluxes between 2008 and 2012.

	Control		+N		+NP		+P		+Ca	
	[mm]	σ	[mm]	σ	[mm]	σ	[mm]	σ	[mm]	σ
2008	1225	(± 17)	1373	(± 10)	1280	(± 13)	1292	(± 14)	1182	(± 18)
2009	1176	(± 19)	1287	(± 25)	1277	(± 22)	1276	(± 27)	1189	(± 22)
2010	1060	(± 12)	1195	(± 14)	1124	(± 17)	1020	(± 20)	1082	(± 23)
2011	1162	(± 22)	1222	(± 18)	1157	(± 12)	1081	(± 23)	1110	(± 20)
2012	1354	(± 18)	1490	(± 16)	1436	(± 15)	1333	(± 22)	1375	(± 16)

	+N		+NP		+P		+Ca	
	RRx	SE	RRx	SE	RRx	SE	RRx	SE
2008	0.11	(±0.03)	0.04	(± 0.04)	0.05	(± 0.08)	–0.04	(± 0.10)
2009	0.09	(±0.06)	0.08	(± 0.10)	0.04	(± 0.14)	0.00	(± 0.12)
2010	0.12	(±0.06)	0.06	(± 0.08)	–0.05	(± 0.09)	0.01	(± 0.10)
2011	0.06	(±0.04)	0.00	(± 0.07)	–0.08	(± 0.11)	–0.05	(± 0.05)
2012	0.10	(±0.07)	0.06	(± 0.08)	–0.02	(± 0.08)	0.02	(± 0.03)

The table shows annual cumulative troughfall and differences among treatments expressed as natural log-response ratios (RRx) between each treatment (n = 4) and the unfertilized control plots (n = 4). Values in brackets indicate plus or minus one average standard deviation (σ) or one standard error (SE) for similar treatments.

element concentrations and their responses to nutrient additions.

Effects of Nutrient Additions on Mean Annual Nutrient Concentrations in Ecosystem Solutions

Cumulative Responses of Element Concentrations in Throughfall after 5 Years

The analysis of element concentrations by linear mixed models (**Figure 4**) revealed a potentially negative, but not significant effect ($p = 0.135$) of separate P additions on TOC concentrations in throughfall, which were 17 ± 7% (SE) lower than in the control treatments. Simultaneous additions of N and P led to marginally higher mean concentrations of DON (13 ± 4%, $p = 0.092$). Separate amendments of Ca did not show significant effects on TOC and DON concentrations. Mean TOC/DON ratios were ca. 10% narrower in all treatments ($p < 0.05$) compared to the control as a result of overall lower TOC and higher DON concentrations in throughfall.

Nitrogen additions generally resulted in increased concentrations of inorganic N species. The NH_4-N concentrations were 7 ± 3% higher than in the control in the plots with separate N additions ($p = 0.080$) and 12 ± 3% higher in the N+P treatment ($p = 0.002$). Nitrate-N concentrations showed a strong response to higher N supply. They were 37 ± 6% higher ($p = 0.001$) if N was separately added and 73 ± 9% higher ($p < 0.001$) if N was added in combination with P. While separate P additions had no effect on NH_4-N concentrations in throughfall ($p = 0.703$), they caused an increased NO_3-N production in the canopy resulting in concentrations which were 22 ± 9% higher ($p = 0.032$) than in the control treatments. These results indicate that higher N supply increases the N richness of the canopy and leads to higher concentrations of inorganic N species in throughfall, and that NO_3-N production and/or leaching in the canopy is stimulated by the addition of P, both separately or in combination with N. Total N concentrations in throughfall seem to be driven by the direct contributions of NH_4-N, while Ca additions did not show any significant effects on inorganic N concentrations in throughfall over 5 years.

Cumulative Responses of Element Concentrations in Litter Leachate after 5 Years

The effects of separate P amendments and of simultaneous N+P additions on TOC and DON concentrations tended to be similar to those already observed in throughfall (**Figure 5**). Mean TOC concentrations were 15 ± 8% lower ($p = 0.121$) in the plots with P amendments and mean DON concentrations 16 ± 6% higher ($p = 0.103$) in the combined N+P treatments compared to the control, but the differences were not significant. Ca additions had no effect on TOC and DON concentrations in litter leachate, while TOC/DON ratios were narrower in the plots to which N was separately added ($p = 0.004$) relative to the control treatments.

Both N and P amendments to the forest soil showed strong and significant effects on inorganic N concentrations in litter leachate compared with the control treatment. Accordingly, NH_4-N concentrations were 16 ± 4% higher ($p < 0.001$) in response to the simultaneous N+P amendments and 23 ± 5% higher ($p < 0.001$) after separate N additions, while NO_3-N concentrations doubled in the N+P treatments and were 3.7 times higher in the plots where N alone was added. Separate additions of P and of Ca did not increase N concentrations in litter leachate, while TN concentrations were driven by inorganic N contributions.

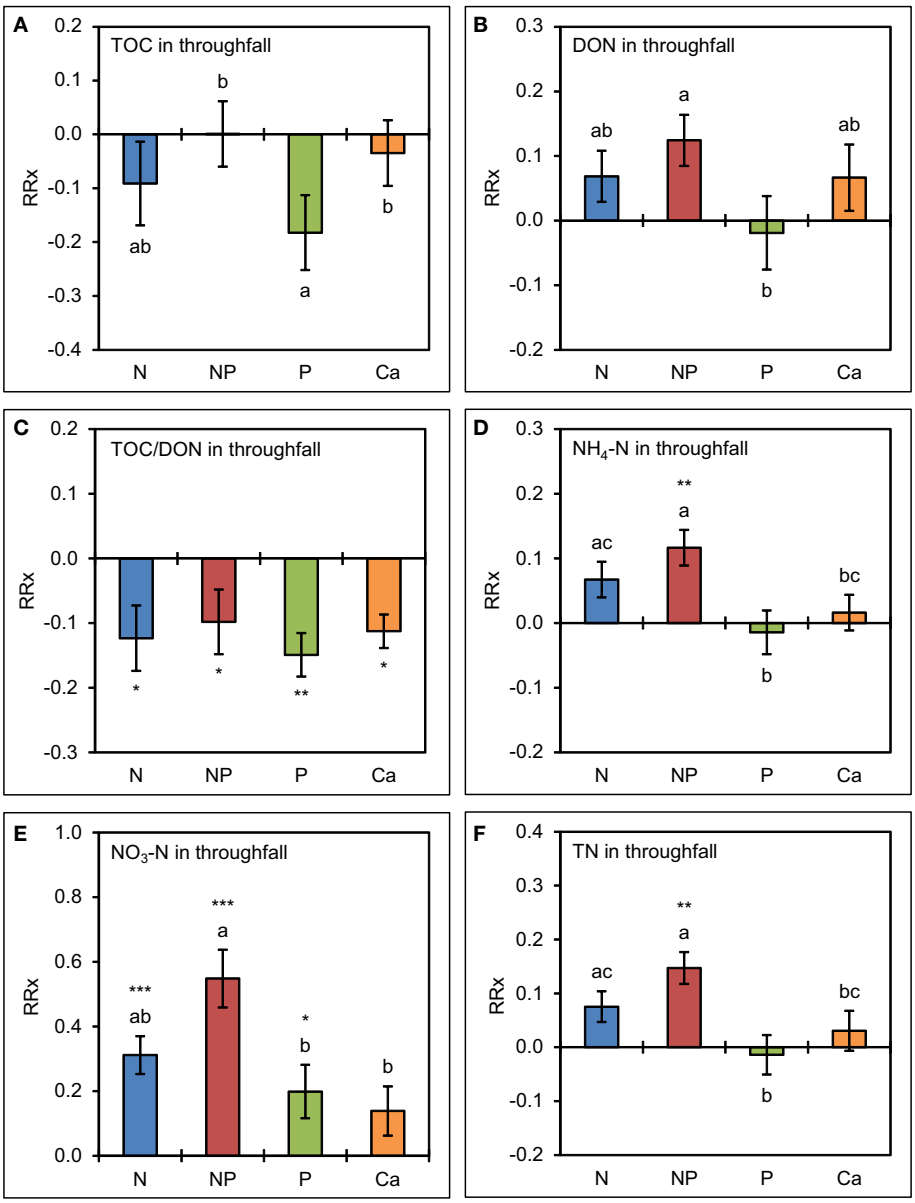

FIGURE 4 | Cumulative effects of nutrient manipulations observed in throughfall after 5 years of nutrient additions. The diagrams show the average responses over 5 years of **(A)** total organic carbon (TOC) concentrations, **(B)** dissolved organic nitrogen (DON) concentrations, **(C)** TOC/DON ratios, **(D)** ammonium-N (NH_4-N) concentrations, **(E)** nitrate-N (NO_3-N) concentrations, and **(F)** total nitrogen (TN) concentrations to nutrient additions expressed as natural log-response ratios (RRx) between the respective treatments ($n = 4$) and the unfertilized control plots ($n = 4$). Error bars indicate plus or minus one standard error. Asterisks indicate significant differences to the control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Values not sharing a common letter are significantly different from each other ($p < 0.05$).

Concentration Time Series in the Control Plots

Temporal Course of Element Concentrations in Throughfall

The TOC concentrations in throughfall decreased by 16% in the unfertilized control plots between the first and the fifth year of NUMEX ($\tau = -0.248$, $p = 0.033$). At the same time, the temporal courses of DON concentrations and of TOC/DON ratios did not show significant trends (**Figure 6**). DON contribution to TN decreased by 20% (**Figure 7**) because of increasing inorganic N richness in the canopy, which is attributed to the rising N deposition from the atmosphere in the research area (**Figure 3**).

Concentrations of inorganic N in throughfall showed a high seasonality and doubled in 5 years ($\tau = 0.328$, $p = 0.005$ for NH_4-N, and $\tau = 0.440$, $p < 0.001$ for NO_3-N). The course of TN concentrations was driven by the strong increase in NH_4-N and NO_3-N concentrations over time, while the pH values of throughfall increased from 5.0 at the beginning of the experiment in 2008 to 5.4 in 2012, which represents roughly a 50% decrease of the proton concentration after 5 years ($\tau = -0.280$, $p = 0.016$).

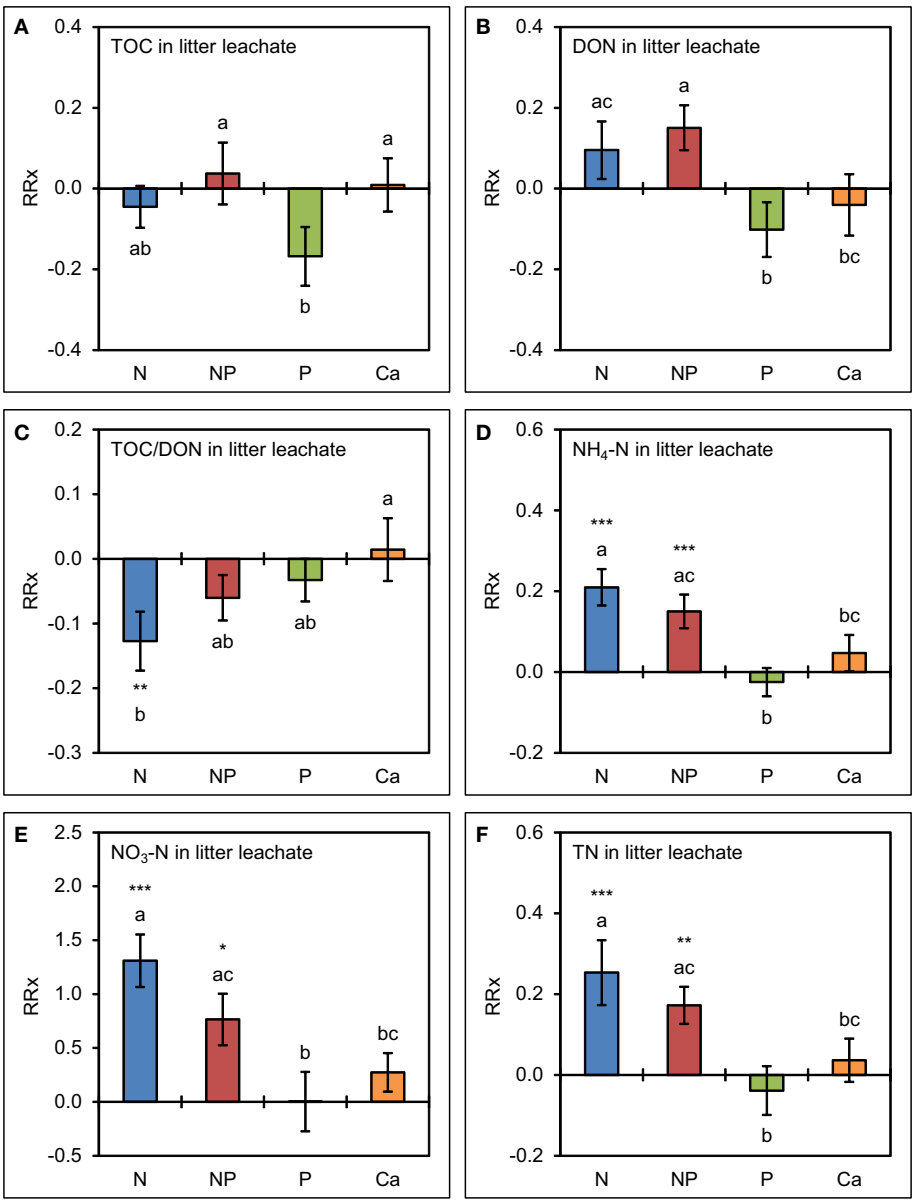

FIGURE 5 | Cumulative effects of nutrient manipulations observed in litter leachate after 5 years of nutrient additions. The diagrams show the average responses over 5 years of **(A)** total organic carbon (TOC) concentrations, **(B)** dissolved organic nitrogen (DON) concentrations, **(C)** TOC/DON ratios, **(D)** ammonium-N (NH_4-N) concentrations, **(E)** nitrate-N (NO_3-N) concentrations, and **(F)** total nitrogen (TN) concentrations to nutrient additions expressed as natural log-response ratios (RRx) between the respective treatments ($n = 4$) and the unfertilized control plots ($n = 4$). Error bars indicate plus or minus one standard error. Asterisks indicate significant differences to the control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Values not sharing a common letter are significantly different from each other ($p < 0.05$).

Temporal Course of Element Concentrations in Litter Leachate

In litter leachate (**Figure 6**), concentrations of TOC and DON showed high seasonality but no overall trend. The pH values of litter leachate did not change ($\tau = 0.072$, $p = 0.536$), so that there was no acidification of the organic layer in spite of the rising N deposition mainly as NH_4^+, which could have been expected to favor acidification of the organic layer because of the release of protons during nitrification (Malhi et al., 1998). Similar to our observations in throughfall, concentrations of NH_4-N varied seasonally and showed a strong positive trend ($\tau = 0.521$, $p < 0.001$). The NH_4-N concentrations of litter leachate in the control plots doubled between beginning of 2008 and end of 2012 while the NO_3-N concentrations were often close to the limit of detection and displayed an increasingly higher variance to the end of the analyzed time series, yet without showing a temporal trend ($\tau = 0.060$, $p = 0.617$).

The concentration ratio of NH_4-N to NO_3-N is approximatively 10:1, which indicates that the system is dominated by NH_4^+ and that there is little nitrification of the

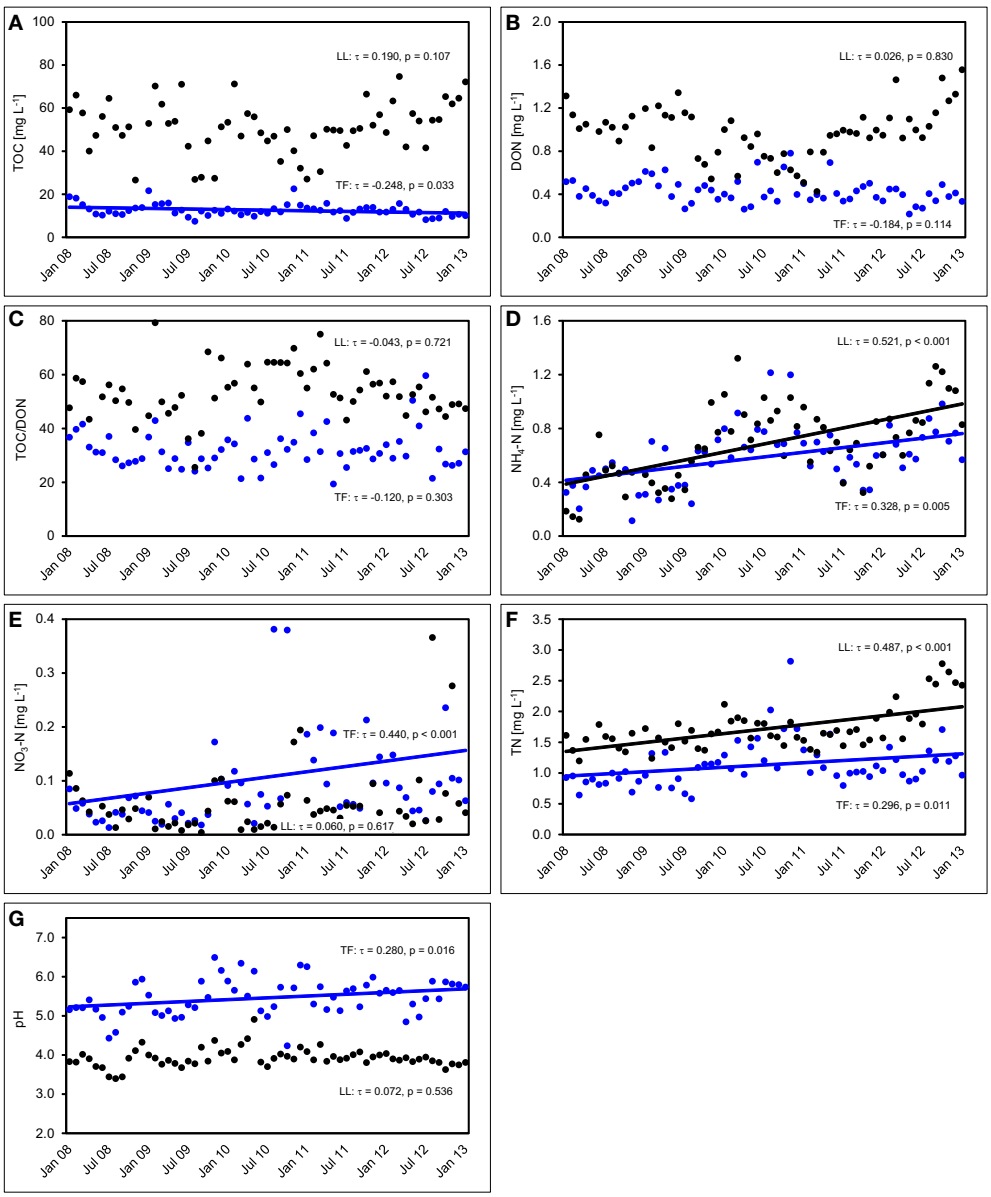

FIGURE 6 | Concentration time series in the control plots between January 2008 and December 2012. The figures show the course of volume-weighted, mean monthly values of **(A)** total organic carbon (TOC) concentrations, **(B)** dissolved organic nitrogen (DON) concentrations, **(C)** TOC/DON ratios, **(D)** concentrations of ammonium-N (NH_4-N), **(E)** concentrations of nitrate-N (NO_3-N), **(F)** concentrations of total nitrogen (TN), and **(G)** pH values in throughfall (TF, blue), and litter leachate (LL, black) of the control plots ($n = 4$) of the nutrient manipulation experiment (NUMEX). Solid lines illustrate significant trends in the dataset ($p < 0.05$).

NH_4-N deposited from the atmosphere in the organic layer. At the same time, TN concentrations increased by 36% ($\tau = 0.487$, $p < 0.001$) between the beginning and the end of the observations, which was attributable to strongly increasing NH_4-N contributions to TN (26 to 44%), while DON contributions to TN dropped from 71 to 53%. NO_3-N contributions remained stable at ca. 3% of TN (**Figure 7**). TOC/DON ratios were lower in throughfall than in litter leachate and showed a high seasonal variation but no temporal trends. In summary, there was a dynamic change of the N cycle in the untreated control plots of our study during the observation period of 5 years, which is mainly attributable to the steadily increasing N deposition from the atmosphere (**Figure 3**).

Effects of Nutrient Additions on Concentration Time Series

Concentration Time Series and Trends in Throughfall

In throughfall of the plots with separate N additions, TOC ($\tau = 0.223$, $p = 0.059$) and DON concentrations ($\tau = 0.248$, $p = 0.033$) increased over the 5 years compared with the control (**Figure 8**). Separate P additions did not cause a response of TOC concentrations in throughfall ($\tau = 0.056$, $p = 0.630$), while

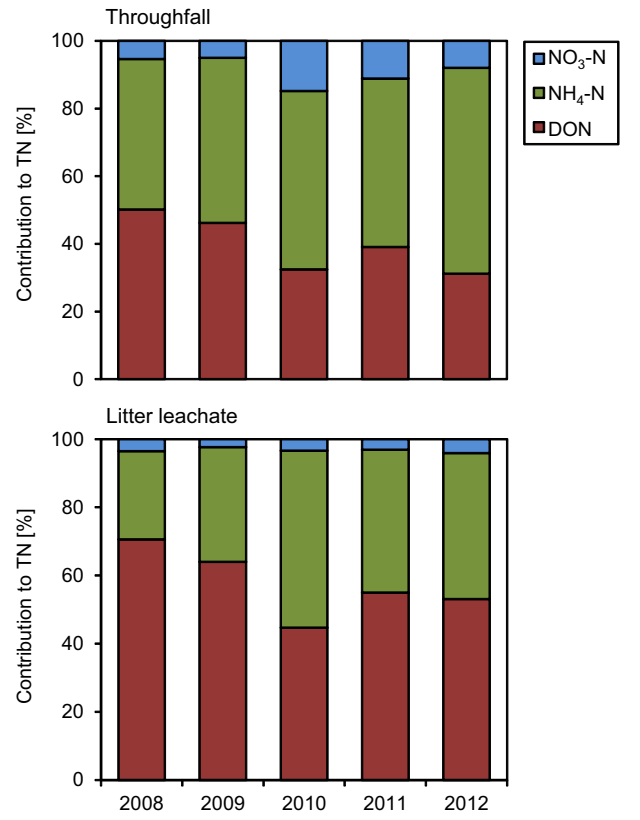

FIGURE 7 | Contributions to total nitrogen (TN) in throughfall and litter leachate between 2008 and 2012. The diagrams show the individual contributions of dissolved organic nitrogen (DON), ammonium-N (NH_4-N), and nitrate-N (NO_3-N) to total nitrogen (TN) in the control plots of the nutrient manipulation experiment (NUMEX).

separate Ca additions led to an ascending, but not significant trend ($\tau = 0.168$, $p = 0.149$) of TOC concentrations in throughfall over time. Combined N+P additions had apparently no influence on the course of TOC ($\tau = 0.136$, $p = 0.243$) and DON concentrations ($\tau = 0.056$, $p = 0.630$) during the observed 5 years. The time series of TOC/DON ratios did not show temporal trends in any of the treatments. After a fast response in the first 2 years, the effects of nutrient additions on TOC and DON concentrations declined and almost disappeared after 5 years.

The concentrations of NH_4-N and NO_3-N in throughfall behaved similarly as in the control plots and followed the positive concentration trends described in **Figure 6** without major deviations. This indicates that most of the added N is retained in the system. This finding is also supported by the positive response of TN concentration time series in throughfall of both the N ($\tau = 0.248$, $p = 0.033$) and N+P treatment ($\tau = 0.216$, $p = 0.063$), although the latter trend was only marginally significant. Steadily increasing TN concentrations in throughfall with time, which represent the sum of organic and inorganic N species, are the effect of a steadily increasing N richness in the canopy because of the fertilizer additions. Ca fertilization did not result in temporal trends of element concentrations in throughfall, beside a marginally significant increase in TN concentrations ($\tau = 0.200$, $p = 0.086$), while the time series of the pH values in throughfall showed seasonal oscillations, but no trend compared with the control treatment.

Concentration Time Series and Trends in Litter Leachate

In litter leachate (**Figure 9**), we observed a strong decrease of the TOC concentrations over time as a result of P ($\tau = -0.322$, $p = 0.006$) and N+P additions ($\tau = -0.372$, $p = 0.002$). The response of the TOC concentrations was strongest in the time series of the combined N+P treatment ($\tau = -0.372$, $p = 0.002$). We did not detect significant responses in the temporal course of TOC concentrations in litter leachate after separate addition of N. Contrary to our expectations based on findings of a coupling of Ca cycle and N leaching losses (Perakis et al., 2006), addition of Ca did not generate a response of the concentrations of N species over time. DON concentrations in litter leachate decreased in the plots with P ($\tau = -0.266$, $p = 0.030$) and simultaneous N+P additions ($\tau = -0.385$, $p = 0.001$), while the decrease was not significant in the plots where N alone was added ($\tau = -0.111$, $p = 0.353$). Similarly to the TOC concentrations, the strongest effect on the time series of DON concentrations was observed in the combined N+P treatment.

There were no significant differences in pH values of litter leachate between the treatments and the control plots over the complete observation period, which confirms that the increased supply of N occurring as NH_4-N did not acidify the organic layer during the observation period. The TOC/DON ratios in litter leachate did not significantly change with time after fertilizer additions, which was similarly observed in throughfall.

In the plots with separate N additions, NH_4-N concentrations in litter leachate followed the same temporal patterns which we had already observed in the control plots, but decreased with time in the plots with separate P ($\tau = -0.398$, $p = 0.001$) and combined N+P additions ($\tau = -0.350$, $p = 0.003$). Furthermore, NO_3-N concentrations rose in the N treatment ($\tau = 0.419$, $p < 0.001$) after 2010, but decreased significantly following separate P additions ($\tau = -0.239$, $p = 0.049$) and simultaneous N and P amendments ($\tau = -0.385$, $p = 0.001$). This indicates a possible regulating role of P availability for the N use efficiency in the study ecosystem.

There was a fast initial positive response of N concentrations in litter leachate to P and N+P amendments. However, in the later course of the experiment, the differences to the control plots decreased (**Figure 9**). This suggests that after a fast increase of N concentrations in response to the N and P additions there was a lag time before the system could make use of the additionally available nitrogen.

We observed no significant effects of inorganic N concentrations in litter leachate in response to Ca additions. Once more, TN concentrations in little leachate were driven by the NH_4-N concentrations, while DON contributions to TN dropped by ca. 18%, as similarly observed in the control plots (**Figure 7**).

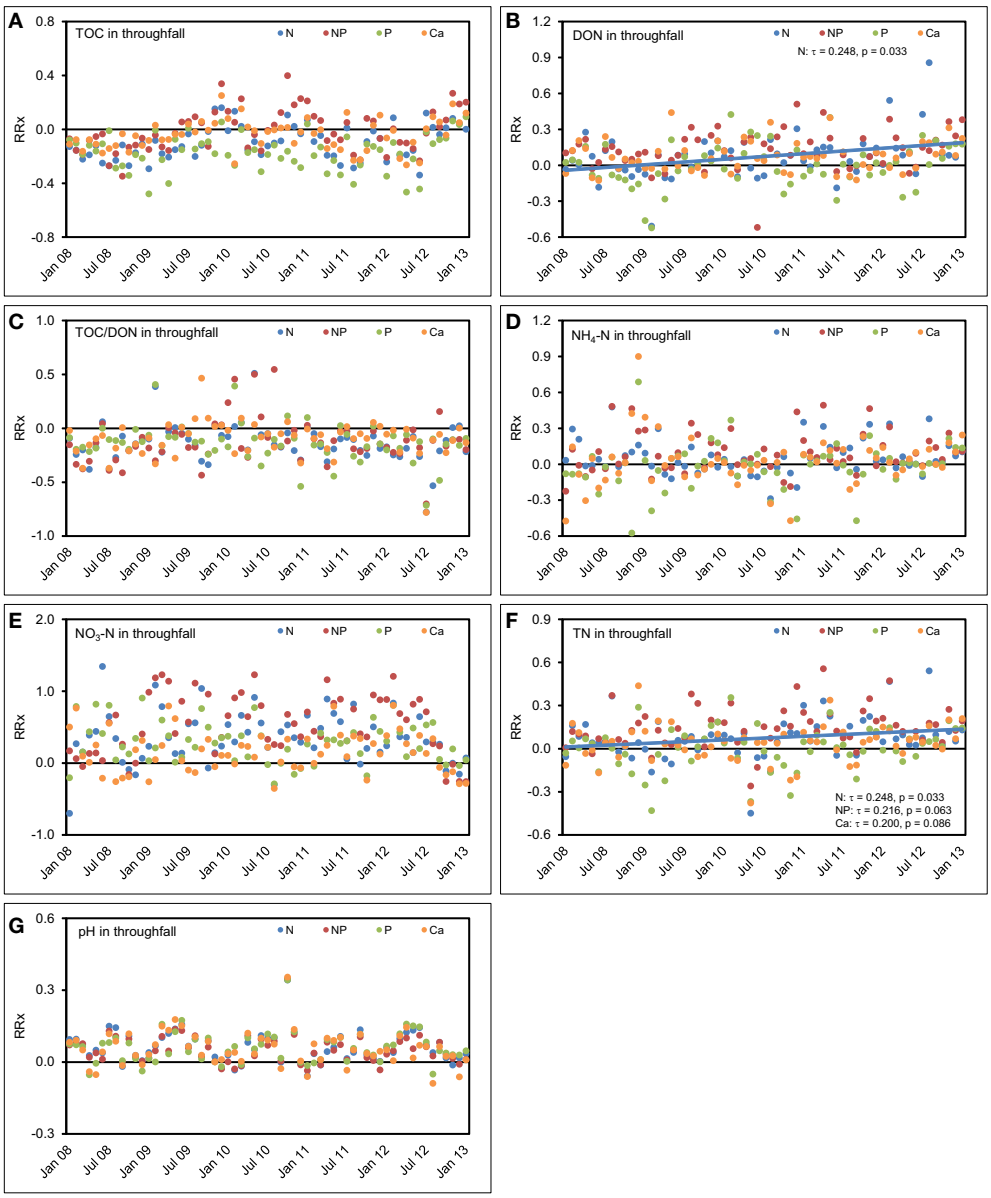

FIGURE 8 | Concentration time series in throughfall between January 2008 and December 2012. The figures show the course of the differences between the four treatments and the control plots expressed as natural log-response ratios (RRx) of **(A)** total organic carbon (TOC) concentrations, **(B)** dissolved organic nitrogen (DON) concentrations, **(C)** TOC/DON ratios, **(D)** concentrations of ammonium-N (NH_4-N), **(E)** concentrations of nitrate-N (NO_3-N), **(F)** concentrations of total nitrogen (TN), and **(G)** pH values in throughfall of the nutrient manipulation experiment (NUMEX). Solid lines illustrate significant trends in the dataset ($p < 0.05$).

DISCUSSION

Effects of Nutrient Addition on N Concentrations in Throughfall and Litter Leachate

Five years after the establishment of NUMEX, we showed that additions of N alone or simultaneously with P generally increased annual mean N concentrations in the above-ground part of the forest water cycle (**Figure 4**). The absence of trends of the log response ratios of inorganic N concentrations in throughfall (**Figure 8**) denotes a fast response of the above-ground part of the water-bound forest N cycle to N and P additions or their combination. This confirms previous findings in the research area, where N return with throughfall was increased (Wullaert et al., 2010) and N concentrations in fine litterfall augmented (Homeier et al., 2012) after 1 year of nutrient additions, but also shows that the response to nutrient additions was highly variable during the 5 years of our observations, which is probably attributable to the variable N input from the atmosphere (**Figure 3**). Increasing inorganic N deposition from

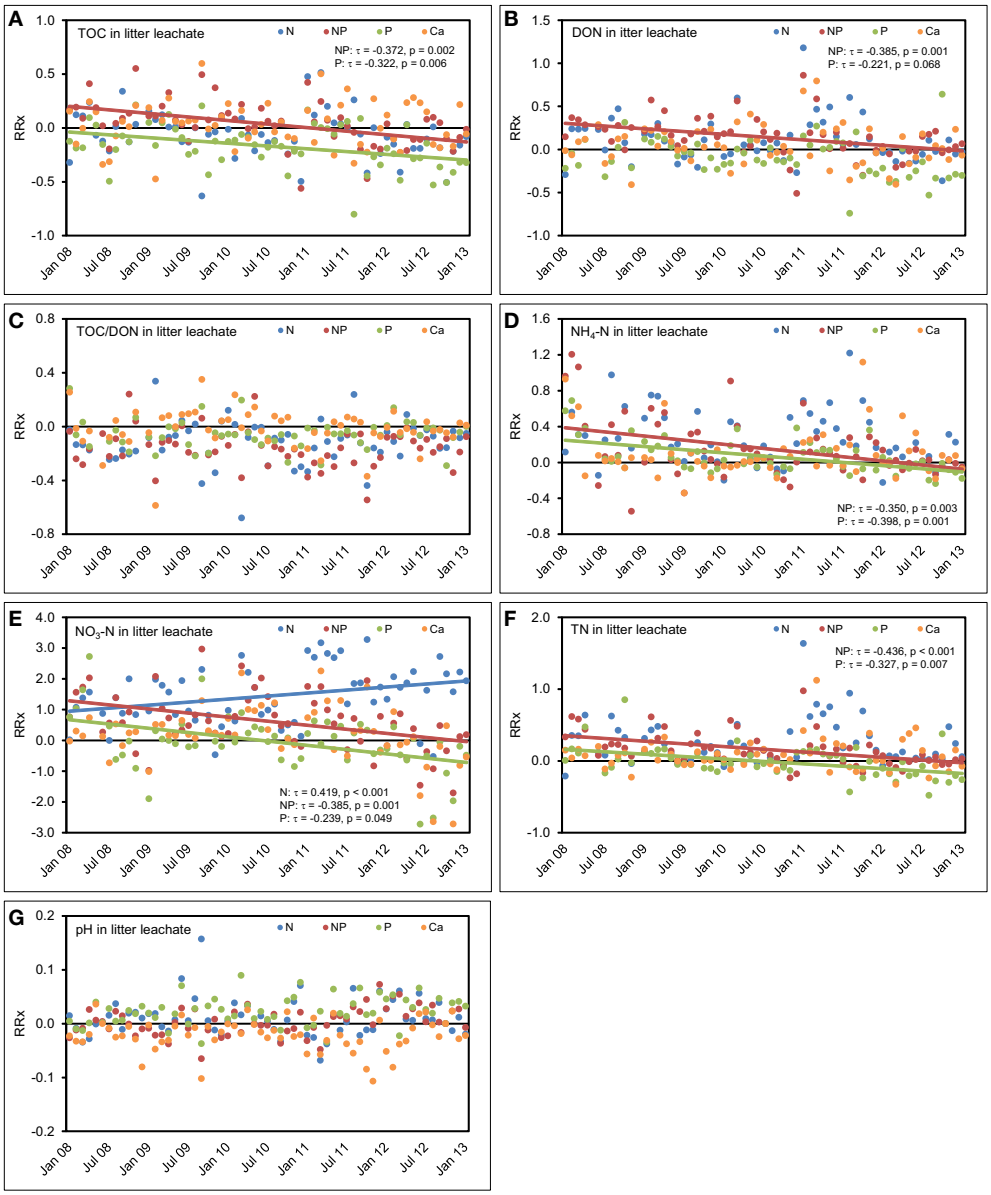

FIGURE 9 | Concentrations time series in litter leachate between January 2008 and December 2012. The figures show the course of the differences between the four treatments and the control plots expressed as natural log-response ratios (RRx) of **(A)** total organic carbon (TOC) concentrations, **(B)** dissolved organic nitrogen (DON) concentrations, **(C)** TOC/DON ratios, **(D)** concentrations of ammonium-N (NH_4-N), **(E)** concentrations of nitrate-N (NO_3-N), **(F)** concentrations of total nitrogen (TN), and **(G)** pH values in litter leachate of the nutrient manipulation experiment (NUMEX). Solid lines illustrate significant trends in the dataset ($p < 0.05$).

the atmosphere, which has already been documented by Boy et al. (2008) and Wilcke et al. (2013a) for our research area, leads to an N enrichment of the canopy, where N could be retained by the abundant epiphytes, which may establish closed nutrient cycles in the associated organic matter accumulations in the canopy (Nadkarni, 1994). Furthermore, N cycling in canopy soils of tropical montane forests was shown to be stimulated by N and P additions to the forest soil, but long-term fertilization may also lead to increased N leaching from the canopy (Matson et al., 2014). The studies of Vance and Nadkarni (1990) and Schwarz et al. (2011) have shown that mineralization of organic matter in the canopy and nitrification on leaf surfaces occur in tropical forests. Overall higher concentrations of NO_3-N in throughfall of the N, P, and N+P treatments (**Figure 4**) denote that nutrient additions to the soil have either favored nitrification in the canopy or increased NO_3-N leaching. The lack of trends in the log response ratios of NH_4-N and NO_3-N concentrations (**Figure 8**) indicates that the nitrification rates in the canopy did not change over time in response to nutrient additions. Yet, more NO_3-N is produced when NH_4-N supply through natural deposition

or fertilization increases, although the nitrification rate remains constant. We argue that N, P, and N+P additions stimulate N uptake by the vegetation, accelerate inorganic N cycling, and increase NO_3-N production in the forest canopy, which consequently leads to increased NO_3-N losses with throughfall.

Reactive N species NH_4^+ and NO_3^- can be easily taken up by microorganisms and plants and thus stimulate biomass production in N-limited ecosystems (Janssens et al., 2010). It was also shown, however, that NO_3-N losses occurred immediately after the first N addition in a lowland tropical forest thought to be N limited (Lohse and Matson, 2005). N and N+P amendments resulted in overall higher inorganic N losses from the forest floor, but showed opposite temporal trends (**Figure 9**). The negative trends of NH_4-N and NO_3-N concentrations in litter leachate denote that P amendments alone or in combination with N reduced initially increased inorganic N leaching from the organic layer over time back to the levels of the control or to even slightly lower levels. While separate P additions did not generate a different response of the annual mean N concentrations from the control treatment (**Figure 5**), the trend analysis revealed that increased P availability leads to a higher N retention in the root zone (**Figure 9**), which denotes a potential P limitation of plants and micro-organisms. Thus, added N could be efficiently used by plants and the microbial community only if P availability was also improved.

When N demands of microbes and plants are exceeded, N leaching from the ecosystem may be further enhanced by long-term availability of excessive N (Law, 2013). Since, it is known that nitrification occurs even at very low pH values in tropical forest soils (Robertson, 1982), rising NO_3-N concentrations with time may also indicate increased nitrification because of drier microclimatic conditions in the organic layer. Accordingly, the increasing NO_3-N leaching which we observed in the plots with separate N additions indicates a shift toward a more open N cycle, where N alone is not a limiting nutrient and, once present in excess, it is removed from the system primarily in NO_3-N form (Matson et al., 1999).

We expected that Ca additions would generate a similar response of the N cycle as the addition of N, as observed by Perakis et al. (2006) in forest ecosystems developed under maritime climate on the Oregon Coast Range. However, we only found a marginally significant positive trend of TN concentrations in throughfall in response to Ca amendments (**Figure 9**). We speculate that the highly variable natural deposition of Ca during the 5 years of our study obscured the expected fertilization effects of Ca additions on N concentrations, or annual addition rates of Ca have been too low to trigger significant responses. Consequently, our hypothesis of a possible coupling of Ca addition and N concentrations in the studied forest cannot be confirmed.

Effect of Nutrient Addition on TOC and DON Concentrations in Throughfall and Litter Leachate

Annual mean TOC and DON concentrations in throughfall responded weakly to nutrient amendments. The overall differences from the control treatments were not significant (**Figure 4**) and only DON concentrations revealed a temporal trend in throughfall during the observation period (**Figure 8**). The absence of overall effects and the slow change over time indicate that TOC exports from the canopy were not influenced by increased nutrient availability. We attribute increasing DON concentrations with throughfall over time to the overall higher availability of reactive N in the canopy which stimulates biomass production (Janssens et al., 2010) and hence indirectly also production of DOM. Hydrophilic DOM contains more N (Qualls and Haines, 1991), is typically mobile (Kaiser and Zech, 1998) and can thus be directly leached to the forest floor. Gaige et al. (2007) also reported higher DON exports with through fall as a result of direct N additions to the canopy. Additionally, DON can be first immobilized by microorganisms in the phyllosphere through biotic processes (Stadler and Müller, 2000) and subsequently leached from the surface of the leaves. This would explain the increasingly higher TN concentrations in the N treatments (**Figure 8**).

In litter leachate, the overall response of TOC and DON concentrations was similar to their response in throughfall (**Figure 5**). However, litter leachate became poorer in TOC and DON with time since the start of the experiment if P was added alone or in combination with N, while N additions did not generate a trend in the response of TOC or DON concentrations (**Figure 9**). A possible reason for decreasing TOC and DON concentrations in litter leachate could be lower pH values resulting in increasing protonation of the DOM, thus reducing its solubility (Curtin et al., 1998). However, acidity did not increase in litter leachate in response to nutrient amendments, possibly as a consequence of generally low nitrification in the organic layer, which has already been reported by Martinson et al. (2012) for our study area. This is consistent with our trend analysis showing that N originating from atmospheric deposition was increasingly leached as NH_4^+ in the litter leachate of the control plots (**Figure 6**).

Moreover, increasing air temperature and unevenly distributed rainfall have allowed for longer dry spells, leading to reduced air and soil humidity in our study area (Peters et al., 2013; Wilcke et al., 2013a), which likely favored mineralization of DOM by microorganisms and led, consequently, to lower TOC and DON concentrations in litter leachate. Similar experiments in tropical forests showed that microbial mineralization of DOM was stimulated by the increased P content of the DOM (Cleveland et al., 2006). Homeier et al. (2012) have already shown that the P pool of the south Ecuadorian tropical montane forest increased in the organic layer after combined addition of N and P. We demonstrate that concurrent improvement of both N and P availability increases the mineralization of TOC and DON in the organic layer likely by stimulating the activity of microorganisms which, in turn, mineralize more DOM. Since this was true only if P was simultaneously added, we conclude that P demands of microorganisms in the organic layer are not completely met.

Our study confirms the importance of the relationships between nutrient supply, leaching, and demands of organisms (Townsend et al., 2011). Because the strongest negative trends of

TOC and DON concentrations in litter leachate always appeared in the N+P treatment (**Figure 9**), this denotes that plants and microbial community can more efficiently use the available N only as a result of the improved P supply. These observations are in our view a strong indication that microbial activity in the organic layer of the studied tropical montane forest ecosystem is co-limited by N and P. This finding is also supported by the apparent accumulation of N in the organic layer at the beginning of the experiment, which is later increasingly leached as NO_3^- in the plots with separate N amendments (**Figure 9**). Delayed NO_3-N losses have already been observed in the soils of a lowland wet forest in Hawaii as a consequence of a high anion exchange capacity (Lohse and Matson, 2005). We argue therefore that availability of P in the organic layer (e.g., through P deposition with Saharan dust) will play a key role in DOM mineralization in the long run by accelerating TOC and DON turnover in the organic layer.

We expected that nutrient additions would result in wider TOC/DON ratios, because of the preferential mineralization of the more hydrophilic, polar fraction of DOM, but our results contradicted our hypothesis and indicated strong opposite effects for all treatments in throughfall (**Figure 4**) and weak opposite effects in litter leachate (**Figure 5**). Narrower TOC/DON ratios may result from (a) an increased DON richness in the canopy and in the organic layer in the case of separate N and combined N+P additions and (b) a reduction of TOC concentrations in response to separate P additions. This may be attributed to a higher phyllosphere activity leading to increased TOC consumption as a consequence of the expected N fertilization effect on canopy and soil microorganisms. Plants and microorganisms can directly assimilate low molecular weight amino acids from DON (Neff et al., 2003), but rising availability of easily assimilable inorganic N species—mainly in litter leachate of the plots with separate N additions—may have diminished the demand of plants and microorganisms for DON, which is instead leached to the mineral soil, leading to narrower TOC/DON ratios in litter leachate.

CONCLUSIONS

Our results demonstrate that N additions alone or in combination with P generally increased mean N concentrations in the above-ground part of the forest water cycle and led to an enrichment of the inorganic species NH_4^+ and NO_3^- in throughfall. Total N concentrations in throughfall increased with time only in the N treatment, indicating rising N richness in the canopy and suggesting a potential P limitation of canopy organisms. Nitrogen additions alone led to rising NO_3-N exports from the organic layer, while separate P and combined N+P amendments reduced NH_4-N and NO_3-N leaching from the organic layer with time. We conclude that increased P availability stimulates N uptake by plants and immobilization by microorganisms, thus reducing N losses from the ecosystem. However, we did not observe evidence of the coupling of Ca and N concentrations in the studied forest ecosystem.

Separate N additions generated a steady increase of DON concentrations in throughfall. Interestingly, TOC exports from the canopy were not affected by nutrient additions. In the organic layer, separate additions of P and simultaneous additions of N and P stimulated mineralization of TOC and DON, while there was no significant response to separate additions of N. In absence of acidification of the litter leachate following N additions, we conclude that the availability of P plays a key role in the mineralization of TOC and DON by accelerating DOM turnover in the organic layer. Our findings contradicted the hypothesis that nutrient additions would lead to wider TOC/DON ratios over time. Rising availability of inorganic N species favored DON leaching to the mineral soil, leading thus to narrower TOC/DON ratios in litter leachate.

Increased supply of P—alone or together with N—will lead to a more efficient use of available N by microorganisms and plants and hence to reduced N concentrations in solutions originating from the organic layer in the long run. Thus, TOC and DON cycling will only change if the availability of N and P is concurrently improved, while NO_3-N leaching from the organic layer will further rise if the N supply alone increases.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Nutrient Addition Modifies Phosphatase Activities along an Altitudinal Gradient in a Tropical Montane Forest in Southern Ecuador

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Atmospheric nutrient deposition and climate change are expected to endanger the diversity of tropical forest ecosystems. Nitrogen (N) deposition might influence nutrient fluxes beyond the N cycle by a concomitant increased demand for other nutritional elements such as phosphorus (P). Organisms might respond to the increased P demand by enhanced activity of enzymes involved in releasing inorganic P from organic matter (OM). Our aims were to assess the effect of (i) climate shifts (approximated by an altitudinal gradient), and (ii) nutrient addition (N, P, N+P) on phosphatase activity (PA) in organic layer and mineral soil of a tropical montane rainforest in Southern Ecuador. A nutrient manipulation experiment (NUMEX) was set up along an altitudinal gradient (1000, 2000, and 3000 m a.s.l.). We determined PA and inorganic and total P concentrations. PA at 1000 m was significantly lower (mean ± standard error: 48 ± 20 μmol p-NP g^{-1} dm h^{-1}) as compared to 2000 and 3000 m (119 ± 11 and 137 ± 19, respectively). One explanation might be that very rapid decomposition of OM at 1000 m results in very thin organic layers reducing the stabilization of enzymes and thus, resulting in leaching loss of enzymes under the humid tropical climate. We found no effect of N addition on PA neither in the organic layer nor in mineral soil, probably because of the low nutrient addition rates that showed ambiguous results so far on productivity measures as a proxy for P demand. In the organic layers of P and N+P treatments, we found decreased PA and increased concentrations of inorganic P. This indicates that the surplus of inorganic P reduced the biosynthesis of phosphatase enzymes. PA in megadiverse montane rainforests is likely to be unaffected by increased atmospheric N deposition but reduced upon atmospheric P deposition.

Keywords: phosphomonoesterase, phosphodiesterase, Ecuador, NUMEX, tropical montane forest, fertilization, nutrient limitation, phosphorus

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INTRODUCTION

Tropical forests comprise essential functions in global processes such as regulating hydrological and climatic cycles, serving as terrestrial carbon stocks and harboring exceptionally high plant diversity. But human activities even affect remote areas where these ecosystems are located (Dixon et al., 1994; Field et al., 1998; Avissar and Werth, 2005). Particularly the Tropics suffer from global

environmental change in terms of climate change and altering nutrient fluxes (Phoenix et al., 2006; Boy et al., 2008; Wright, 2010). Up to now, the consequences of atmospheric and climate change for the tropical forests are poorly understood. Increasing nitrogen (N) depositions (Galloway et al., 2004; Wilcke et al., 2013) will have implications on virtually all processes of net primary production in tropical forests since they are known for limited nutrient supply (Vitousek and Howarth, 1991). Atmospheric phosphorus (P) deposition was even suggested to be an important nutrient source for tropical forest ecosystems (Okin et al., 2004). Lowland tropical forests grow on highly weathered soils with less bioavailable P and more strongly bond P fractions, whereas N deficiency plays a minor role (Walker and Syers, 1976). Tropical mountain areas have younger soils and are characterized by lower N availability (Tanner et al., 1998). The Andean forest in Southern Ecuador is known for its exceptionally high plant diversity (Homeier et al., 2008) and is considered for conservation priorities (Myers et al., 2000). It has been shown that these forests respond to nutrient addition (Tanner et al., 1998; Cavelier et al., 2000; Homeier et al., 2012; Matson et al., 2014) and the direction and dimension of the response will depend decisively on soil composition and nutrient supply of the specific zone (Walker and Syers, 1976; Phoenix et al., 2006). However, knowledge about belowground processes and responses of the forest ecosystems to altering nutrient fluxes and climatic changes are scarce (Soethe et al., 2006; Homeier et al., 2008).

In the Ecuadorian montane forest, mainly N and P are supposed to limit aboveground productivity (Wullaert et al., 2010; Homeier et al., 2012), decomposition and microbial activity (Iost et al., 2008; Krashevska et al., 2010, 2012). Plants can adapt in multiple ways to P limitation, like reduced growth, modified structure, and function of roots, changes in P allocation and metabolism of P (Wilcke et al., 2008; Tischer et al., 2014). Soil organic matter is an important source for P in particular in tropical montane forests, where soils are usually poor in nutrients and their availability depends largely on the mineralization of organic matter. While desorption or dissolution contribute to release P in mineral soil, it can be considered negligible in the organic layer (Walker and Syers, 1976; Wilcke et al., 2008). Accordingly, the organic layer can be seen as the key component of P release which, contrary to most temperate forests, harbors most of the root mass (Wullaert et al., 2010). In these ecosystems, most soil P is bond in detritus as organic P and therefore inaccessible to plants. During the mineralization process, phosphatase enzymes hydrolyze organically bond P and release inorganic P in the soil solution, which can be taken up by plants and microbes (Frossard et al., 1995; Hinsinger, 2001). Phosphatases are responsive to P demand and plant and microorganisms compensate the insufficient supply through phosphatase exudation (McGill and Cole, 1981; Allison and Vitousek, 2005). Hence, phosphatases play an important role in maintaining and controlling the rate of P cycling in forest ecosystems. They can originate from several sources like plant roots, fungi, protozoa, microorganisms, and in extracellular form in soil (Nannipieri et al., 2011). Phosphatase activity depends on soil properties like pH, water content, soil organisms, presence of growing plants, soil composition, and nutrient supply. Some of these properties can be expected to respond to environmental change. Altitudinal gradients are ideal means to study the response of tropical montane forests to climate change since the predicted changes correspond the climatic gradient of 3000–1000 m. For the montane forest in Southern Ecuador, increasing temperature and decreasing moisture are predicted (Bendix et al., 2013; Wilcke et al., 2013). Temperature and moisture, both affecting pedogenesis and nutrient cycling, change with elevation (Tanner et al., 1998; Unger et al., 2010). They are key factors in controlling P availability in soil because they influence mineralization, microbial activity, and impact plant growth- related P demand (Grierson and Adams, 2000; Leirós et al., 2000).

With increasing altitude, the C:N and N:P ratio in soil increases, meaning that N and P become more important for organism growth (Wilcke et al., 2008). N addition is supposed to enhance phosphatase activity in P-limited and N-rich sites because N can be invested in phosphatase production and it has a growth-stimulating effect (Olander and Vitousek, 2000). So even P-limited systems might respond to N deposition with increasing growth (Treseder and Allen, 2000). A global meta-analysis of fertilization effects on phosphatase enzymes revealed a positive effect of N addition on phosphatase activity (Marklein and Houlton, 2012). Similarly, N addition stimulated phosphatase activity in the N-limited site (Olander and Vitousek, 2000). In contrast, P addition had a negative effect on phosphatase activity (Marklein and Houlton, 2012) and a negative relationship between nutrient supply and enzyme activity was observed for P (Olander and Vitousek, 2000). Phosphatase exudation in soil mostly occurred under P limiting conditions in soil (Tate, 1984).

To study the effect of environmental change in terms of climate and atmospheric deposition, a nutrient manipulation experiment (NUMEX) with moderate N and P addition along an altitudinal transect started in 2008. At three different altitudes, fertilized blocks in a full-factorial randomized design were established in an old-growth tropical montane forest in Southern Ecuador (Wullaert et al., 2010; Homeier et al., 2012). We measured the activity of two different types of phosphatases, acid phosphomonoesterase (PMA), and phosphodiesterase (PDA). We based our research on the previous findings about the study site and the assumption of an N and P co-limitation for this Andean tropical forest (Wullaert et al., 2010; Homeier et al., 2012).

We hypothesized that (i) biologically mediated P release in soil is constrained by organism activity associated with moisture and temperature and therefore, phosphatase activity in soil decreases with increasing altitude; (ii) N addition stimulates biomass production resulting in an increased P demand; the increased need for P is reflected in a higher phosphatase activity; (iii) since microorganisms and plants can easily take up inorganic P added as fertilizer, the need to invest resources for phosphatase exudation will be reduced. Hence, phosphatase activity will be lower in the P treatment as compared to the control.

MATERIALS AND METHODS

Study Site

The study sites included three different altitudinal levels ranging from about 1000–3000 m a.s.l., located in the Cordillera Real, an eastern range of the Southern Ecuadorian Andes. The lowest site was located at 990–1100 m (S 4°7′ W 78°58′), the second at 2020–2120 m (S 3°58′ W 79°04′), and the highest at 2900–3050 m (S 4°7′ W 79°11′) a.s.l. (Homeier et al., 2013). The slope gradient ranged from about 26° at 1000 m to 31° at 2000 m. The area is a declared biodiversity hotspot located in protected areas, with more than 800 tree species described and the vegetation type is classified as an evergreen lower montane forest (Homeier et al., 2008). The annual mean temperature decreases from 19.4°C (1000 m) to 9.4°C (3000 m), annual precipitation increases from 2230 mm (1000 m), and 1950 mm (2000 m), to 4500 mm (3000 m; Moser et al., 2007). Paleozoic metamorphic schists and sandstones with some quartz veins form the parent material for soil development. At 1000 m, parent material comprises deeply weathered granitic rock of the Jurassic Zamora granitoide formation (Litherland et al., 1994). In addition, the thickness of the organic layer ranges from very thin at the 1000 m location (mean 4.8 cm) to massive accumulation of organic material increasing with height up to 44 cm (Moser et al., 2008).

Sampling Design

We conducted the sampling at the NUMEX sites, a fully randomized two-factor block design experiment with four blocks. Each block included a 20 × 20 m plot per treatment with an unfertilized control, N, P, and a combined treatment N+P. Within each plot, six 2 × 2 m subplots were marked randomly. To handle potential nutrient leaching from fertilized blocks, the control was in the uppermost and the N+ P treatment in the lowermost plot. Fertilization started in February 2008 with moderate nutrient addition of 50 kg ha^{-1} $year^{-1}$ of N (as urea) and 10 kg ha^{-1} $year^{-1}$ of P (as $NaH_2PO_4 \cdot 2\ H_2O$) divided into two applications per year. For further description of the experimental set up, see Homeier et al. (2012). In October 2014, we took 48 soil samples (3 subplots × 4 treatments × 4 blocks) with a soil corer of 20 cm length and 5 cm width by choosing randomly three subplots out of six. Soil cores were separated into horizons of the organic layer and the uppermost 5 cm of mineral top soil. Samples were transported to the scientific station of San Francisco (ECSF), Ecuador, homogenized and stored at ambient temperature for 8 h respectively, 32 h maximum between sampling and analyses. We measured PMA and PDA in the organic layer the following day, and 2 days after sampling in mineral soil, respectively. We determined soil pH in a deionized water suspension using glass electrodes at a water:soil ratio of 1:2. The remaining samples of the organic layer and mineral soil were oven-dried at 60°C and 105°C, respectively, to determine soil moisture (=gravimetric water content) and later on transferred to the University of Tübingen, Germany, for further analysis of total and inorganic P concentrations.

Chemical Analysis

Acid phosphatase activities were measured according to the original method by Tabatabai and Bremner (1969) and Eivazi and Tabatabai (1977) for PMA; and Browman and Tabatabai (1978) for PDA – both slightly modified by Margesin and Schinner (1994). We removed stones and debris greater than 2 mm from the homogenized soil samples and additionally plant roots from mineral soil samples. We used 1.0 g of soil to determine enzyme mediated p-nitrophenol (pNP) release in μg after 1 h incubation at 37°C and for the replicates. As substrate, p-nitrophenyl for PMA respectively bis-p-nitrophenyl for PDA was added in a buffered phosphate solution. We used sodium hydroxide to extract and color pNP released by PMA, respectively alkaline Tris solution for pNP released by PDA. The incubated soil solution was filtered and the pNP concentrations determined photometrically at 400 nm with a spectrophotometer (SpectroDirect, Lovibond, Germany). The results were expressed as μmol pNP per gram dry matter per incubation time of 1 h. The concentration of pNP in samples and control was calculated from the calibration curve with calibration standards containing 0.1, 2, 4, 6, 8, 10 g/l of pNP.

Total P of the organic layer was extracted using acid pressure digestions (Kuo, 1996) in a microwave system (Start 1200, MLS, Germany). An aliquot of the sample was milled and we used 0.5 g for the microwave digestion with HNO_3 and H_2O_2. Concentrations of total P in the digests were determined by means of an ICP-OE (5300 DV, Perkin Elmer, Germany).

We determined Bray-extractable inorganic P (P_i) concentrations in the organic layer and in mineral soil according to Bray and Kurtz (1945). A solution containing HCl and NH_4F was added to 2 g dried soil (soil:solution ratio of 1:10). The samples were shaken on a horizontal shaker for 5 min and subsequently filtered through P-free filters. P_i concentrations were measured photometrically with a continuous flow analyzer (AutoAnalyzer, Bran&Luebbe, Germany).

We calculated specific enzyme activity for the organic layer by dividing PMA of the organic layer through the total P concentration of the respective plot.

Statistical Analysis

All statistical analysis were conducted in IBM® SPSS® Statistics Version 22. We used the Levene test for confirming homogeneity and Shapiro-Wilk test for normal distribution. Altitudinal effects of all investigated parameters were compared using the control plots of each study in a univariate ANOVA in case of parametric data with Tukey-HSD as post-hoc test. In case of non-parametric data, a Kruskal–Wallis test for independent samples was performed. In order to compare differences in measured variables between the control and each addition treatment, the effects of N and/or P addition were expressed as natural-log transformed response ratios [RR_x = ln (measured value in nutrient addition treatment/measured value in the control)] (Hedges et al., 1999; Elser et al., 2007). RR_x-values were tested in a pairwise student-*t*-test against zero to verify significant differences if comparing to the control value. To investigate linkages between the investigated parameters, a

two-sided Pearson correlation and Spearman correlation was performed, the latter in case of non-normally distributed data.

RESULTS

Effect of Altitude and Experimental Nutrient Addition on Phosphatase Activity in the Organic Layer and Mineral Soil

At all sites, phosphatase activity in the organic layer was significantly higher than in mineral soil. PMA and PDA were closely correlated in the organic layer ($r = 0.74$, $p < 0.001$) and mineral soil ($\rho = 0.83$, $p < 0.001$) with PMA being higher than PDA (**Figure 1**). We found a significant difference in soil moisture in the organic layer between 1000 and 2000 m, 3000 m (mean % ± SE: 1000 m, 0.61 ± 0.03 < 2000 m, 0.80 ± 0.04; 3000 m, 0.81 ± 0.04). Soil moisture ranged between 24 and 84% and between 19 and 73% for the organic layer and mineral soil, respectively. In the organic layer, PMA correlated positively with soil moisture ($r = 0.86$, $p < 0.001$). We found the lowest PMA in the organic layer of control plots at 1000 m altitude whereas no difference was observed between 2000 and 3000 m altitude. There were no significant differences in PMA of mineral soil among altitudes (**Figure 1A**) nor were there any for PDA among the sites for both organic layer and mineral soil (**Figure 1B**). No relationship was found between phosphatase activity and total P concentrations in the organic layer ($r = 0.18$, $p > 0.05$).

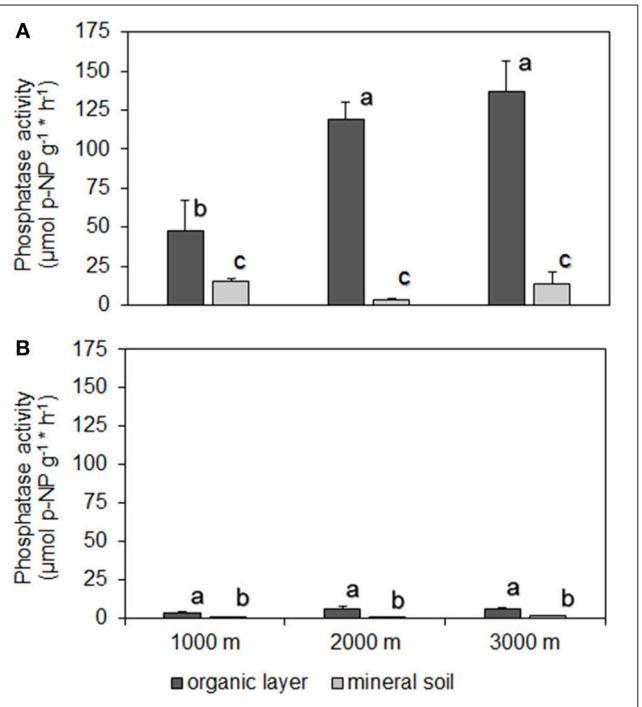

FIGURE 1 | Activities of phosphomonoesterases (A) and phosphodiesterases (B) (mean ± 1 SE) in organic layer and mineral topsoil (0–5 cm) along the altitudinal gradient (1000–3000 m) of NUMEX. Significant differences between activities (univariate ANOVA, $P < 0.05$) are denoted with letters (a > b > c).

Analyses revealed that fertilizer treatments had no effect on pH of the organic layer (mean pH 4.1, range pH 3.6–5.9) or mineral soil (mean pH 3.8, range pH 3.4–5.2). Along the altitudinal gradient, PMA in the organic layer of the P treatment were significantly lower as compared to the control (**Figures 2A,B,C**). For PDA this applied to the 1000 and 3000 m sites (RR_x ± SE: 1000 m, −1.05 ± 0.19; 2000 m, −0.93 ± 0.22). For the N+P treatment, PMA in the organic layer was significantly lower compared to control at 1000 m (**Figure 2A**), the same held true for PDA at the 3000 m (RR_x ± SE: −1.05 ± 0.29). In mineral soil at 1000 m altitude, we found a significantly decreased enzyme activity in the P and N+P treatments if compared to the control for PMA (**Figures 2D,E,F**) and PDA (RR_x ± SE: P treatment −0.66 ± 0.06; N+P treatment −1.00 ± 0.24). No significant differences were found in the N treatments at all altitudes, neither the organic layer nor mineral soil except for PMA at 3000 m, where activity was significantly increased as compared to the control (**Figure 2F**).

Effect of Altitude and Experimental Nutrient Addition on Specific Phosphatase Activity in the Organic Layer

Total P concentrations ranged from 450 to 868 mg per kg dry organic matter (**Table 1**). There was no difference among the control plots of the different altitudes, but the effect of fertilization on total P concentrations in the organic layer depended on the altitudinal gradient. We found a significant increase of total P concentration in the P and N+P treatment as compared to the control at 2000 m and 3000 m altitude (mean± SE: P treatment 2000 m 868 ± 42; 3000 m 844 ± 40; N+P treatment 2000 m 783 ± 16; 3000 m 705 ± 83 mg per kg dry mass). We observed significantly decreased specific PMA in the organic layer of P and N+P treatments of all sites if compared to the control (**Figure 3A**). At 1000 and 3000 m altitude, we found significantly lower specific PDAs for the P and N+P treatments (**Figure 3B**). The N treatment had no significant effect on specific phosphatase activities in the organic layer of any of the experimental sites.

Effect of Altitude and Experimental Nutrient Addition on Bray-Extractable P_i Concentrations

Bray-extractable inorganic P (P_i) concentrations of all plots ranged between 24 and 348 mg per kg dry organic matter and 2–72 mg per kg dry mineral soil (**Table 1**). Highest concentrations of Bray-extractable P_i on control plots were found in the organic layer at 2000 m altitude whereas no difference was observed between 1000 and 3000 m altitude (mean ± SE: 1000 m 45 ± 9; 2000 m 129 ± 11; 3000 m 69 ± 7 mg per kg dry mass). In the organic layer, Bray-extractable P_i concentrations differed significantly from the control at the 2000 m site in the N+P (RR_x ± SE: 0.47 ± 0.11) and P treatment (RR_x ± SE: 0.66 ± 0.12) and in the P treatment at 3000 m altitude (RR_x ± SE: 0.80 ± 0.08). We found no significant effects of nutrient addition on P_i concentrations in the mineral soil.

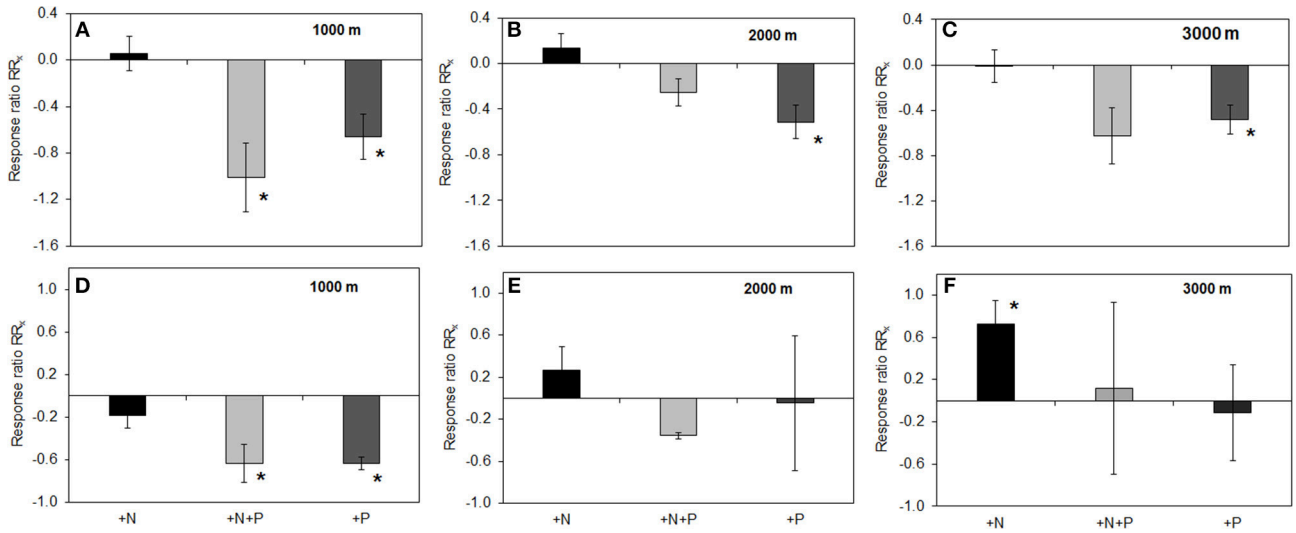

FIGURE 2 | Effects of nutrient addition on phosphomonoesterase activity in the soil. All values represent the effects as natural-log transformed response ratios (RR_x) in which the activity in the fertilized treatments is divided by its value in the control treatment and then ln-transformed. RR_x-values are given for altitudes for organic layer **(A)** 1000, **(B)** 2000, **(C)** 3000 m, and mineral soil **(D)** 1000; **(E)** 2000; **(F)** 3000 m. Error bars indicate ± one standard error. Data of the control treatment (mean ± 1 SE) is given in **Figure 1**. Asterisks indicate values significantly different from zero (*t*-Test, $P < 0.05$).

TABLE 1 | Characteristics of the soil at the three NUMEX sites.

Altitude (m)	Organic layer thickness (cm)	C/N	Total C (g/kg)	Total N (g/kg)	Total P (mg/kg)	P_i (mg/kg)	pH
1000 m							
Organic layer	4.8[a]	19.2[b]	430[d]	22[d]	450 ± 28	45 ± 9A	4.7
Mineral soil	–	9.3[c]	6.0[c]	0.6[c]	220 ± 36	6 ± 1	5.0
2000 m							
Organic layer	305[a]	17.5[b]	410[d]	19[d]	527 ± 22	129 ± 11B	4.6
Mineral soil	–	17.2[c]	10.6[c]	0.7[c]	53 ± 5	7 ± 1	4.3
3000 m							
Organic layer	435[a]	23.7[b]	420[d]	15[d]	525 ± 47	69 ± 7A	4.6
Mineral soil	–	14.2[c]	9.6[c]	0.7[c]	175 ± 68	25 ± 16	4.1

The values represent the mean of the four control plots ± standard error if given. [a] Moser et al., 2008; [b]Wolf et al., 2011; [c]Homeier et al., 2013; [d]Iost et al., 2008. Different capital letters indicate significant differences among altitude.

DISCUSSION

According to previous findings, PMA and PDA were correlated with PMA being constantly higher than PDA (Frankenberger and Dick, 1983; Rastin et al., 1988; Trasar-Cepeda et al., 2000). PDA and PMA are coupled as they operate successively, hydrolyzing diester and monoester bonds, respectively. A generally low PDA in acidic soils might be linked to the dominance of fungi or of sorption of the enzyme (Turner and Haygarth, 2005; Nannipieri et al., 2011). Concurrent with literature, phosphatase activity in the organic layer was much higher than in mineral soil because of the generally higher microbial activity in the organic layer (Speir and Ross, 1978).

Contradictory to our hypothesis, PMA in the organic layer at 1000 m were significantly lower as compared to higher altitudes (**Figure 1A**). This result is surprising because the lower part of the altitudinal gradient is supposed to be limited by P (Homeier et al., 2010; Krashevska et al., 2010; Wullaert et al., 2010; Martinson et al., 2013) raising the need of organism to mobilize P e.g., by enzyme exudation. As tropical soils are generally poor in P, the organic layer is the predominant source for P mobilization. Several explanations might be considered for this finding: altitudinal shifts of (i) root biomass, (ii) organic matter available as a substrate for enzymatic hydrolyses, and (iii) characteristics of the organic layer. Belowground root biomass increased slightly with increasing altitude indicating that higher phosphatase activity observed at 2000 and 3000 m sites might be attributable to an increased plant root surface (Moser et al., 2008). However, it remains unclear whether the root surface as a whole was involved in enzyme exudation. Furthermore, we consider increased enzyme exudation by roots at higher altitudes unlikely given the reduced P demand induced by N limitation in conjunction with decreased aboveground productivity (Wilcke et al., 2008; Krashevska et al., 2010). Total P concentrations in the organic layer represent organically bond P and thus, degradable and potentially available substrate for phosphatase enzymes. Total P concentrations in the organic layer did not differ among altitudes neither did the C:N ratio in such a way, that it could explain these differences (**Table 1**). Therefore, we infer that substrate availability for the enzymes cannot be used as an explanation for differences in PMA. Temperature controls enzyme activities both directly by affecting enzymatic reaction kinetics and indirectly by influencing microbial proliferation (Sinsabaugh et al., 1991). Because PMA increased with altitude and thus, was opposite to expectations, we assume that

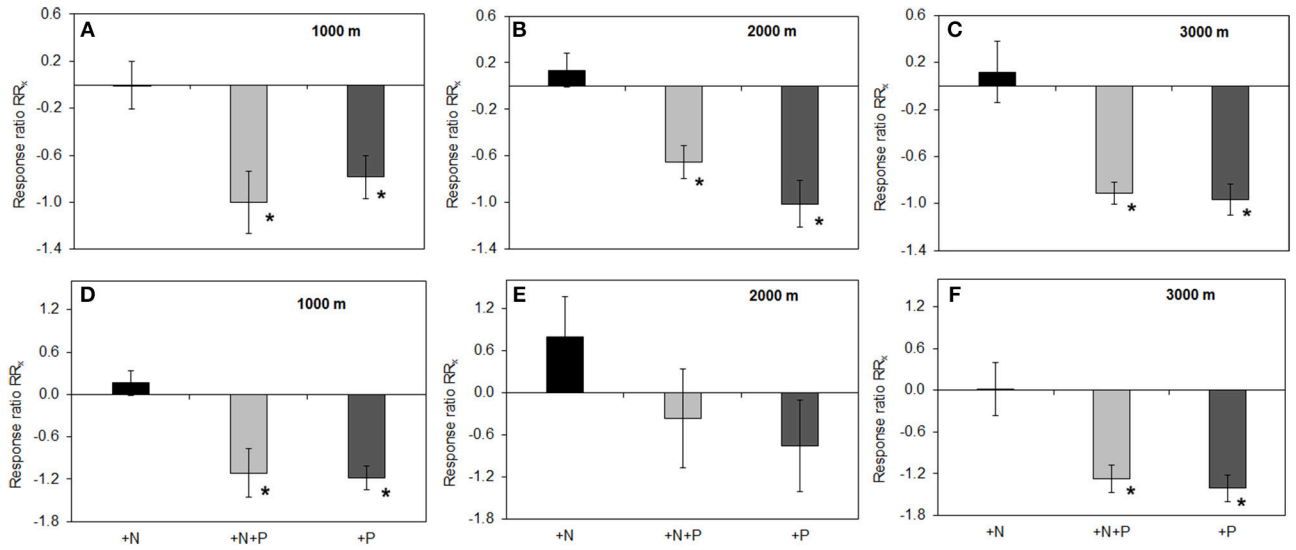

FIGURE 3 | Effects of nutrient addition on specific phosphomonoesterase activity in soil. RR_X-values are given for phosphomonoesterase **(A–C)** and phosphodiesterase **(D–F)** in the organic layer of all altitudes **(A)** 1000, **(B)** 2000, **(C)** 3000 m, and mineral soil **(D)** 1000, **(E)** 2000, **(F)** 3000 m. For illustrational details, see caption of **Figure 2**. Asterisks indicate values significantly different from zero (*t*-Test, $P < 0.05$).

temperature is of minor importance for phosphatase activity. The relatively small temperature difference of ∼6°C between the lowest and the highest altitude of our study sites (Moser et al., 2008) might render temperature effects on enzyme activities negligible. Soil moisture influences the growth of microorganisms and therefore the amount of enzymes being exudated beforehand the measurement of phosphatase activity. Our results revealed decreased PMA in the organic layer combined with a significantly lower moisture content at 1000 m compared to 2000 and 3000 m altitude. Along the altitudinal gradient of our study, reduced soil moisture at the lowest altitude was related to reduced air humidity caused by higher temperature and lower precipitation as compared to higher altitudes (Moser et al., 2008). Therefore, mechanistically moisture could explain higher enzyme activities at higher altitudes. Furthermore, at 1000 m altitude, the characteristics of the organic layer at 1000 m were quite different from the higher altitudes, partly it was not existent or large particles of organic matter were dispersed widely over gritty mineral topsoil. These characteristics of the organic layer might lead to altitudinal differences in the contribution of free versus stabilized enzyme activities to PMA. Measured enzyme activities comprise those of stabilized extracellular enzymes absorbed and stabilized by surface-reactive soil particles (Nannipieri et al., 2011). As the texture of the organic layer at 1000 m was quite coarse compared to higher altitudes, stabilized extracellular enzymes might contribute less to PMA as compared to 2000 and 3000 m altitudes. In contrast, free extracellular enzymes can be rapidly degraded. Therefore, the higher contribution of free extracellular enzymes and subsequent rapid degradation might lead to an underestimation of PMA at 1000 m altitude.

Elevated N availability in the N treatments showed no effect on neither PMA nor PDA, except for mineral soil at the 3000 m site (**Figure 2**). These results contradict our hypothesis and previous findings of a positive effect of N fertilization on PMA (Treseder and Vitousek, 2001; Gress et al., 2007). The assessment of net primary production in a megadiverse forest is a challenging task but so far, previous studies indicated a positive influence of N addition for the Ecuadorian montane forest ecosystems (Wullaert et al., 2010; Homeier et al., 2012). Elevated N availability induced by fertilizer addition led to higher aboveground biomass production in terms of increased tree diameter and leaf litter production. Similarly, total organismic activity increased after N and N+P addition (Homeier et al., 2013). However, other measures contributing to net primary production such as leaf litter production and basal area increment did not increase after N addition (Homeier et al., 2012). Therefore, it remains unclear whether or not N addition increased the nutrient demand, which might explain the non-existent effect of N addition on phosphatase activities. Even if the N addition-induced increase in components of net primary productivity can be verified, these might be counteracted by other variables controlling phosphatase activity. For example, a negative effect of N addition was reported for soil microbial biomass (Homeier et al., 2012) and standing fine root biomass (Homeier et al., 2013). Less microbes and a reduced fine root biomass are associated with a decreased surface for enzyme exudation and tended to occur when nutrients are sufficiently available (Bloom et al., 1985; Chapin et al., 1987). N addition in NUMEX generally resulted in small effects and a positive or negative influence depended on the investigated entity. These complex and non-uniform reactions hamper general deductions of increased organismic P demand caused by N addition, which might be the reason for unchanged phosphatase activity after N addition in our study. Furthermore, the N addition effects observed in other studies might be associated with two- to more than fourfold N

fertilization rates compared to NUMEX (e.g., 220 kg N ha^{-1} yr^{-1}, Campo et al., 2007; 100 kg N ha^{-1} yr^{-1}, Treseder and Vitousek, 2001).

The addition of P reduced phosphatase activity in the organic layer along the gradient (**Figures 2A–C**) whereas in mineral soil, we observed P and N+P treatment effects on PMA only at the 1000 m site (**Figure 2D**). The observation of the P treatment effect for the organic layer is even more depicted when enzyme activity is related to its substrate (here: organic P). The specific PMA and PDA showed a clear reduction of activity in the P and N+P treatment (**Figure 3**) thus, illustrating that the P treatment effects are independent of substrate availability. In an environment where P availability is low, mineralization of organic P and therefore phosphatase production is driven by P demand (Clarholm, 1993; Olander and Vitousek, 2000). In P addition treatments, the fertilizer represents a direct nutrient resource, which can be easily taken up by microorganisms and plants. Accordingly, at 2000 and 3000 m altitude, P addition increased inorganic P availability in the organic layer, which is also corroborated by other studies (Clarholm, 1993; Olander and Vitousek, 2000). The missing effect of P addition on Bray-extractable P_i concentrations in the organic layer at 1000 m altitude might be explained by the thin organic layer promoting percolation of added P to mineral soil. Another explanation might be related to fast turnover rates of organic matter at this altitude associated with accelerated organismic (e.g., microorganisms and plants) immobilization of excess P. Nevertheless, the negative effect of P fertilization on PMA and PDA occurred independent of altitude. If we compare the fertilizer application ratio of N and P in the treatment plots (N:P = 5) with the litter N:P ratio measured in unfertilized control plots (mean: 20–31, see Homeier et al., 2012), we can infer a disproportionately high P addition compared to N. This indicates that in an ecosystem with a natural P deficiency already moderate P augmentation exceeds P demand and leads to a reduced enzyme exudation, thus the system might then be constrained by other factors. Readily available P_i and the reduced energy investment into enzyme exudation seemed to explain increased productivity caused by P addition (Homeier et al., 2012). Therefore, phosphatase activity in megadiverse montane rainforests is likely to be unaffected by increased atmospheric N deposition but reduced upon atmospheric P deposition.

CONCLUSION

Environmental changes, such as nutrient deposition, influence above- and belowground processes and by this, modify structure and functioning of ecosystems. Counterintuitive results on increasing phosphatase activity with increasing altitude were likely attributable to an underestimation of phosphatase activity at 1000 m caused by loss of non-stabilized enzymes. Irrespective of altitude, N addition did not influence phosphatase activity whereas P addition reduced phosphatase activity. Therefore, P (and potentially other nutritional elements) are more important for organism growth than N along the altitudinal gradient. Indirect effects of atmospheric N deposition are negligible for tropical montane forests whereas these ecosystems respond rapidly to direct effects of P deposition. Although no increase in atmospheric P deposition was observed for our study site (personal communication Wolfgang Wilcke), such direct effects might be relevant for tropical montane rainforests prone to atmospheric P deposition e.g., through aerosol input deriving from deserts or phosphate mining areas.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Increases in Soil Aggregation Following Phosphorus Additions in a Tropical Premontane Forest are Not Driven by Root and Arbuscular Mycorrhizal Fungal Abundances

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Tropical ecosystems have an important role in global change scenarios, in part because they serve as a large terrestrial carbon pool. Carbon protection is mediated by soil aggregation processes, whereby biotic and abiotic factors influence the formation and stability of aggregates. Nutrient additions may affect soil structure indirectly by simultaneous shifts in biotic factors, mainly roots, and fungal hyphae, but also via impacts on abiotic soil properties. Here, we tested the hypothesis that soil aggregation will be affected by nutrient additions primarily via changes in arbuscular mycorrhizal fungal (AMF) hyphae and root length in a pristine tropical forest system. Therefore, the percentage of water-stable macroaggregates (> 250 μm) (WSA) and the soil mean weight diameter (MWD) was analyzed, as well as nutrient contents, pH, root length, and AMF abundance. Phosphorus additions significantly increased the amount of WSA, which was consistent across two different sampling times. Despite a positive effect of phosphorus additions on extra-radical AMF biomass, no relationship between WSA and extra-radical AMF nor roots was revealed by regression analyses, contrary to the proposed hypothesis. These findings emphasize the importance of analyzing soil structure in understudied tropical systems, since it might be affected by increasing nutrient deposition expected in the future.

Keywords: arbuscular mycorrhizal fungi, tropical forest, Ecuador, soil aggregation, fertilization, global change

INTRODUCTION

Soils represent a complex system due to the interactions of roots, fungi, fauna, and bacteria embedded within the soil structural environment. The hierarchical structure of micro- and macroaggregates in soils provides microhabitats, but also is important for gas exchange and water infiltration, protection against erosion, biogeochemical cycling, and especially the protection of soil

organic carbon (C) (Six et al., 2000a; Jastrow et al., 2007). Soils represent one of the largest terrestrial C pools, which is of particular interest in the light of future global change scenarios (Kimble et al., 1990; Batjes, 1996). Tropical systems, in particular, play an important role as C sink, since tropical forests hold an estimated 20% of the world's terrestrial vegetation and soil C pool (Jobbagy and Jackson, 2000; Jimenez and Lal, 2006). However, tropical forests are highly endangered by deforestation, land use change, fertilization but also increased nutrient deposition by anthropogenic activities (Gullison et al., 2007; Galloway et al., 2008; Mahowald et al., 2008). Increases in nutrient deposition have been shown to affect plant diversity, ecosystem productivity, soil community composition as well as nutrient cycling (Treseder, 2008; Bobbink et al., 2010; Isbell et al., 2013; Wilcke et al., 2013b; Camenzind et al., 2014), and thus also directly or indirectly may have an impact on soil aggregation processes.

Studies analyzing the impact of nutrient additions on soil aggregation traditionally strongly focus on agricultural systems (e.g., Wang et al., 2011; Guo et al., 2012; Ortas and Lal, 2012). By contrast, to our knowledge only very few experiments have been conducted in natural systems, covering a limited range of ecosystem types like temperate grasslands (Van Groeningen et al., 2002; Wilson et al., 2009) or dry tropical savanna (Tripathi et al., 2008). The majority of these studies report positive effects of nutrient additions on soil aggregation, often accompanied by increases in root and arbuscular mycorrhizal fungal (AMF) hyphal abundance (Latif et al., 1992; Nobrega et al., 2001; Wilson et al., 2009; Alguacil et al., 2010). The latter are important factors in the process of aggregate formation, though abiotic factors likewise may play an important role: Fertilization studies focusing on abiotic soil parameters also demonstrated interrelations with pH and the proportion of exchangeable cations and electrostatic binding agents (Denef et al., 2002; Graham et al., 2002).

Generally, the formation of soil structure is a complex interaction of biotic and abiotic factors (Tisdall and Oades, 1982). A wide range of parameters involved in this process has been revealed—e.g., soil organic matter content, extracellular polysaccharides, humic substances, pH, and electrostatic binding agents, roots, fungal hyphae, macrofauna, microarthropods, or microbial biomass (Jastrow and Miller, 1991; Denef et al., 2002; Six et al., 2004)—though the relative importance of respective factors depends on soil type and study system (Six et al., 2000b; Denef et al., 2002; Denef and Six, 2005). Especially in highly weathered and acidic tropical soils pH and related Fe and Al contents, which affect electrostatic interactions and mineral-mineral bonding capacities, play an important role (Denef et al., 2002; Six et al., 2002; Denef and Six, 2005). However, across biomes, roots and fungal hyphae, especially mycorrhizal hyphae, have been shown to be one of the main factors involved in macroaggregate formation (Miller and Jastrow, 1990; Jastrow et al., 1998; Rillig and Mummey, 2006). Roots may indirectly affect aggregate stability by impacts on soil water regimes, support of soil microbial communities and dead root decomposition, but also directly via physical enmeshment of soil particles and the exudation of organic cementing agents (Angers and Caron, 1998; Hallett et al., 2009). Likewise, mycorrhizal fungal hyphae have been shown to have effects either indirectly, via influences on soil microbial communities, plant growth and root architecture as well as rapid hyphal turnover as C source, but also directly by physical hyphal enmeshment and the secretion of extracellular compounds (Rillig et al., 2002; Leifheit et al., 2014b), even though the precise mechanisms remain unknown.

Beside their predominant role in the formation of soil macroaggregates, AMF and roots may indeed be an important factor mediating changes in soil structure in the context of increased nutrient deposition by anthropogenic activities, since their abundance is affected by changes in nutrient availability. The main role of AMF—characterized as obligate biotrophic plant symbionts associated with more than 80% of land plants (Wang and Qiu, 2006)—represents improved nutrient uptake for the plant in exchange for photosynthetic C (Smith and Read, 2008). Thus, due to their involvement in nutrient uptake, roots and AMF can be affected by nutrient additions as predicted by the functional equilibrium model (Ericsson, 1995; Johnson, 2010): Under the assumption that plants invest more into structures associated with the acquisition of the most limiting resource, the release of soil nutrient deficiency by nutrient additions will result in an allocation shift toward aboveground structures. Therefore, a net negative effect of nutrient additions on root and AMF abundance is predicted, as has been shown in several experimental approaches mainly conducted in temperate areas (reviewed in Treseder, 2004). However, a large variability of possible outcomes was found also in response to nitrogen (N) vs. phosphorus (P) additions, mainly depending on the primary soil nutrient status (Treseder and Allen, 2002). In this context, the frequent observation of positive responses in AMF abundance is mainly explained by a primary nutrient limitation of the fungus itself (Bolan et al., 1984; Olsson et al., 1997; Johnson et al., 2003).

In this study, we aimed at expanding the knowledge on soil aggregation in a so far understudied biome, the pristine tropical forest. Effects of N and P additions on soil aggregation (measured as percentage of water-stable macroaggregates (WSA) and mean weight diameter (MWD) determined by dry-sieving) were analyzed in relation to biotic factors, namely root and AMF abundance, as well as abiotic factors in a tropical premontane forest located in southern Ecuador (Bendix et al., 2013). This study is part of a multidisciplinary nutrient manipulation experiment (NUMEX), which investigates the effects of moderate nutrient additions on this diverse and fragile ecosystem, in the context of increased nutrient depositions expected in the future (Phoenix et al., 2006; Mahowald et al., 2008; Wilcke et al., 2013a). Detailed responses in intra- and extra-radical AMF abundance as well as root length to N and P additions across an elevational transect in this area have been analyzed previously, also including amongst others this study site (own unpublished data). However, interrelations with other soil processes have not been evaluated. Here, we tested the hypothesis that soil aggregation will be affected by nutrient additions via indirect effects of changes in AMF (especially the extraradical part) and root abundance. Additionally, changes in basic soil properties were analyzed to include other potential influencing factors.

MATERIALS AND METHODS

Study Area

The study area is part of the Podocarpus National Park in the Cordillera Real, the eastern range of the South Ecuadorian Andes (Beck et al., 2008; Bendix et al., 2013). Plots are located in the Bombuscaro Area (4°11′S, 78°96′W) at 990–1100 m a.s.l. close to the city of Zamora, in the province of Zamora-Chinchipe. This area is part of the "Tropical Andes" hotspot of biodiversity (Myers et al., 2000) characterized by its outstanding number of plant species (Homeier et al., 2008) as well as of other organism groups, e.g., birds (Orme et al., 2005) and moths (Brehm et al., 2005). The forest is characterized as evergreen premontane rainforest (Homeier et al., 2008) with a mean annual precipitation of 2230 mm $year^{-1}$ and a mean annual temperature of 19.4°C without pronounced seasonality (Moser et al., 2007). The rainforest at this altitude gets up to 40 m high, with common tree families of Fabaceae, Melastomataceae, Moraceae, Myristicaceae, Rubiaceae, and Sapotaceae (Homeier et al., 2013). The soil is characterized as Dystric Alumic Acrisol developed over deeply-weathered granitic rock (Graefe et al., 2008; Martinson et al., 2013). At this elevational site no organic layer is formed on top of mineral soil and soil texture is described as sandy loam. Furthermore, soils are characterized by a pH of 4.6, C/N ratios of 9.3 (± 0.4) (total C-values of 6.0 (± 0.1) mg g^{-1}) and bulk densities of 0.84 (± 0.1) g cm^{-3} (Martinson et al., 2013).

Experimental Design

Within the pristine forest a nutrient manipulation experiment (NUMEX) was set up, adding N and P in a two-factor randomized block design (Homeier et al., 2012). Nutrient additions started in 2008, with relatively low nutrient applications of 50 kg ha^{-1} $year^{-1}$ of N (as urea) and 10 kg ha^{-1} $year^{-1}$ P (as $NaH_2PO_4 \cdot H_2O$) split into two applications per year. Four blocks were set up, each including one 20 × 20 m plot per treatment (control (Ctr), N, P, and NP). Additionally, within the plots six subplots of 2 × 2 m were randomly installed to facilitate coordinated sampling schemes.

In May 2010 a preliminary survey on soil structure was conducted, based on composite samples taken from each plot. For this purpose samples of the upper 5 cm of soil were taken from three randomly chosen subplots with a soil corer (Ø 5 cm), litter was removed. Samples from each plot were mixed, resulting in an overall amount of 16 samples—four per treatment.

In February 2013 again soil samples were taken from each plot, this time keeping samples originating from three different subplots separate to increase resolution and account for the strong spatial variability, resulting in an overall amount of 48 samples. Soil was sampled from the upper 15 cm of soil with a soil corer (Ø 5 cm), litter was removed.

Soil samples were immediately dried at 40°C, transferred to the Freie Universität Berlin and stored at room temperature.

Soil Structure

The percentage of water-stable macroaggregates (WSA) was determined using an Eijkelkamp wet-sieving apparatus (Eijkelkamp Agrisearch Equipment, Giesbeek, the Netherlands) following the descriptions developed by Kemper and Rosenau (1986). Five grams of dry soil (10.0 g were used in the case of soil samples collected in May 2010) were rewetted by capillary action—after thorough examination the insertion of 5.0 g (carefully taken to represent an adequate subsample of the soil core) turned out to give better results due to the relatively small sieve sizes. After 5 min of sieving, the amount of water-stable macroaggregates (> 250 µm) was determined based on soil material kept on the 250 µm mesh—after an additional correction for coarse matter (Leifheit et al., 2014a). Additionally, the mean weight diameter (MWD) was calculated based on soil dry-sieving, dividing soil structure in its different size classes using a series of stacked sieves (2 mm, 1 mm, 250 µm, 53 µm) (Kemper and Rosenau, 1986; Barto et al., 2010). The resulting MWD represents the sum of proportions of soil material kept on every sieve size class, multiplied by the corresponding average size class (the highest average size class was estimated as 3.5 mm). The following formula was used:

$$MWD = \sum_{i=1}^{n} D_n W_n$$

where D is the mean diameter of each size fraction (in mm), W the proportion of total soil weight occurring in this fraction and n the number of size fractions.

AMF and Root Abundance

For root length analyses, we used soil samples taken at the same time and adjacent to soil cores determined for AMF and soil structure analyses. These samples were transferred to University of Göttingen and stored at 4°C. For root length analyses, samples were soaked in water and adhering soil material was removed using a 0.25 mm sieve. Live fine roots were separated under the stereomicroscope based on color, root elasticity, and the degree of cohesion of cortex, periderm and stele (Persson, 1978; Leuschner et al., 2001). Root length (of live roots) was analyzed using WinRhizo (version 2007, Regent Instrument Inc., Quebec, Canada).

The percentage of AMF root colonization was quantified based on roots stained with 0.05% Trypan Blue by a modified staining protocol (Phillips and Hayman, 1970; Camenzind and Rillig, 2013). Roots were extracted with tweezers from soil and a standardized subset of roots was stained (approximately 30 root pieces of 1–2 cm). AMF root colonization was counted at 200x magnification under a stereomicroscope using the magnified intersections method by McGonigle et al. (1990). Besides the percentage of AMF root colonization, based on root length analyses a value for total AMF colonized root length was calculated.

Hyphae were extracted from 8.0 g of soil based on a modified aqueous filtration extraction method (Bardgett, 1991). Sodium hexametaphosphate solution (35 g L^{-1}) was added to rewetted soil to detach hyphae from soil material for 1 h. Soil material was decanted on a 20 µm mesh (this mesh size was proven to avoid hyphal loss (Camenzind and Rillig, 2013) and transferred to 200 ml Erlenmeyer flasks. A defined amount of supernatant

was mounted on a filter paper (pore size $< 0.45\,\mu m$) by vacuum filtration and stained with 0.05% Trypan Blue. Hyphal length was counted with a grid-line intersect method at 200x magnification under a stereomicroscope (Rillig et al., 1999). AMF hyphae were defined as non-regularly septate hyphae with characteristic unilateral angular projections, that are stained dark- to light-blue by Trypan Blue (Mosse, 1959); all other hyphae were categorized as non-AMF hyphae.

The neutral lipid fatty acid (NLFA) 16:1ω5 has been shown to be a good signature of extra-radical AMF (Olsson et al., 1997; Olsson, 1999; Olsson and Johansen, 2000). Therefore, we extracted neutral lipid fatty acids from 5.0 g of soil following the protocol of van Aarle and Olsson (2003). Neutral lipids were purified from other lipids by silica column fractionation (Bond Elut, Varian Inc., Palo Alto, CA, USA) with chloroform as carrier, and subjected to a mild alkaline methanolysis. The abundance of NLFA 16:1ω5 was quantified on a gas chromatograph with a flame ionization detector and a 50 m HP5 capillary column, as described by Frostegård et al. (1993).

Soil Properties

Total soil C and N contents were determined on milled soil with an Elemental Analyzer (EuroEA, HekaTech, Germany).

Total concentrations of P, Ca, Al, Fe, K, Mg, and Zn as well as soil pH were analyzed on separate soil samples taken in February 2013 (important elements which were included in this study were preselected by principal component analysis, selecting for ecologically relevant variables and excluding collinear predictors (Caruso et al., 2012; Camenzind et al., 2014). Samples were obtained from all six subplots (taken with a soil corer of 3.5 cm diameter), pooled separately for each plot and transferred to University of Tübingen and Freie Universität Berlin for further analyses. Here, the soil material was dried at 40°C. Element concentrations were determined based on milled soil using a microwave (Start 1500, MLS, Germany) pressure digestion with HNO_3 and H_2O_2 as digestion agents. Base elements were determined with an Element Analyzer (ICP-OES 5300 DV, Perkin Elmer, Germany). Soil pH was analyzed in a 1:5 v:v suspension of soil and deionized water.

Statistical Analyses

All statistical analyses were conducted in R version 3.1.2 (R Core Team, 2014). In order to account for the underlying block design, linear mixed-effects models were applied including Block and Plot as random effects. The function lme() from the package *"nlme"* was used (Pinheiro et al., 2014), fitting the model by maximizing the restricted log-likelihood (Zuur et al., 2009). All models were tested for the underlying assumptions of normality and homogeneity. In case these were not met, data were log, or square root transformed. Effects of nutrient additions on WSA, MWD and measured biotic and abiotic factors were analyzed by two-way linear mixed effects models, with N and P as fixed factors. For graphical illustrations, one-way analyses were included to assess single treatment effects in comparison to control plots. Linear correlations among WSA (or MWD) and respective biotic and abiotic predictor variables were determined using linear mixed-effects model. In case of certain abiotic variables being only present at plot level, the arithmetic mean of WSA (and MWD) per plot was calculated and used for correlation analyses. In order to account for the simultaneous impact of interacting variables on soil aggregation, multiple linear regression analyses were conducted. Here, only biotic variables, which were all measured at the same scale (subplot level), were included in the analyses. To select for the most important variables, stepwise model selection by backward elimination was performed based on the Akaike's Information Criterion (AIC). Regression analyses again were based on linear mixed effects models in order to include Block and Plot as random effects.

RESULTS

The amount of WSA was significantly affected by P additions in samples from 2010 and 2013 (**Figure 1**, **Table 1**). In 2010 effects were strongest in the NP treatment (average increase of 13.1%), whereas in 2013 both, P and NP additions similarly affected the amount of WSA (average increase of 4.1 and 3.9%, respectively) (**Figure 1**). Effect size clearly differed among different sampling dates, which seems to be driven by differences among control plots: In 2010 average WSA in control plots was 82.3 ± 3.3%, whereas in 2013 it was 90.9 ± 1.4%. However, variability was much higher in soil samples collected in 2010 (**Figure 1**).

MWD was on average 2.34 ± 0.1 mm (only control samples included), though these values were not coarse matter corrected. MWD was not significantly affected by nutrient additions, however, in both P treatments there was a positive trend (**Figure 1**).

AMF clearly represented the dominant mycorrhizal type in this system, though colonization rates were comparably low, with an average of 25 (± 4.8)%. AM hyphal length was on average 12.4 (± 1.6) m cm^{-3} $soil^{-1}$ and the amount of AMF biomarker NLFA 16:1ω5 was 2.08 (± 0.17) nmol cm^{-3} $soil^{-1}$. AMF intra- and extra-radical abundance was in general positively affected by P additions (**Table 1**). The amount of the AMF biomarker NLFA 16:1ω5 was significantly positively affected by P additions [average amount in the P and NP treatment, respectively: 2.8 (± 0.2) and 2.3 (± 0.2) nmol cm^{-3} $soil^{-1}$], but also the percentage of AMF root colonization showed a marginally significant positive response [average amount in the P and NP treatment, respectively: 29.0 (± 4) and 34.3 (± 5)%]. By contrast, N additions exerted no or even weak negative effects, as observed in the case of AMF hyphal length (**Table 1**). Root length was not affected by nutrient additions [average root length in the control treatment: 0.76 (± 0.16) cm cm^{-3} $soil^{-1}$].

The total amount of phosphorus in the soil was positively affected by P additions, whereas none of the other measured abiotic parameters showed a response (**Table 1**).

In order to find correlations of WSA (and MWD) with biotic and abiotic factors simple as well as multiple regression analyses were conducted. As indicated by simple linear regression, none of the biotic factors was significantly related to the amount of WSA (**Table 2**), and also not to MWD (data not shown). Furthermore, stepwise multiple regression analyses based on AIC model selection revealed no biotic factor correlated with

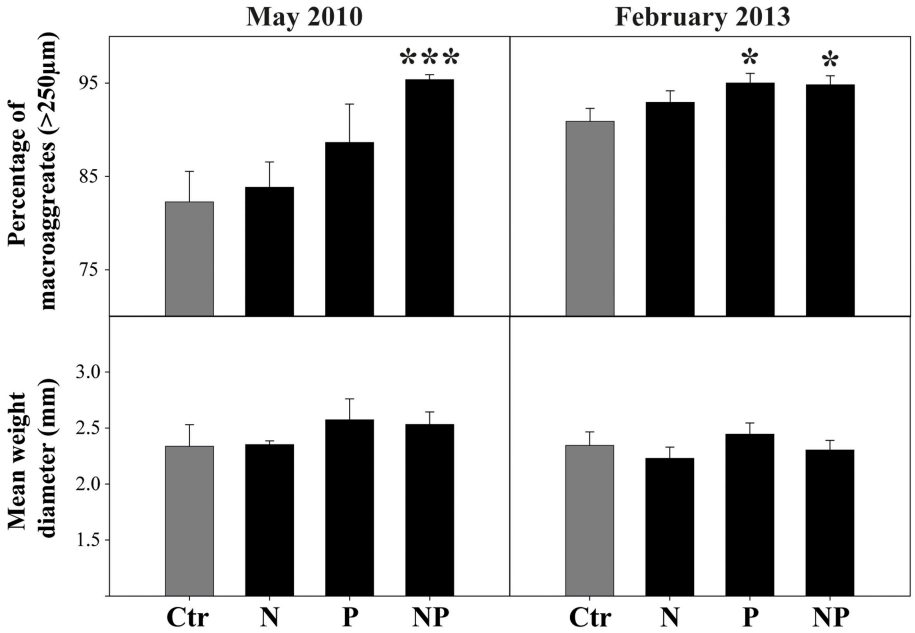

FIGURE 1 | Responses of the amount of water-stable macroaggregates as well as soil mean weight diameter to N and P additions in a tropical premontane forest soil. Bars represent mean values, error bars the respective standard error. Asterisks indicate significant differences compared to the control treatment (one-way linear mixed-effects model; $P < 0.05$).

the amount of WSA, with the lowest AIC obtained by the null model. However, concerning abiotic factors, regression analyses revealed a significantly negative correlation of WSA and C:N ratio, driven by the positive correlation of WSA and total N values, since total C was only marginally significantly correlated with WSA (**Table 2**). Furthermore, total amounts of P also showed a marginally significant positive correlation with WSA (**Table 2**).

DISCUSSION

In summary, in this pristine tropical premontane forest P additions increased the amount of WSA in soil, whereas N additions alone did not exert an effect. Concomitant analyses of mainly biotic, but also some abiotic factors did not reveal a correlation with biotic factors, but only a negative relation of WSA and soil C:N ratios as well as a correlation with treatment associated increases in total amounts of P in the soil. Furthermore, the increase in WSA was not reflected in an increase in MWD, which potentially indicates that these changes do not solely affect the number of macroaggregates, but rather their stability.

In general, the increase in WSA following nutrient additions is in line with most findings from previous fertilization studies. As reported here, P additions have been shown to increase soil aggregation (Nobrega et al., 2001; Gil et al., 2009; Alguacil et al., 2010; Ortas and Lal, 2012), but also N additions (Latif et al., 1992; Tripathi et al., 2008; Wilson et al., 2009; Guo et al., 2012) as well as the application of multi-component fertilizers (Denef et al., 2002; Hati et al., 2006; Huo et al., 2008). As proposed here, many studies focused on the indirect impact of AMF hyphal length and root abundance in this context. For example, irrespective of the study system Wilson et al. (2009; natural field experiment—temperate grassland), Alguacil et al. (2010; agricultural field experiment—tropical savanna) and Nobrega et al. (2001; greenhouse experiment—tropical Oxisol) explained the observed increase in soil aggregation by the concurrent positive effect of nutrient additions on AMF abundance. Other studies related the observed changes in soil aggregation to root abundance (Latif et al., 1992; Jung et al., 2011) or both (Nobrega et al., 2001). However, here we did not find any direct correlation of either AMF intra- or extra-radical abundance or root length with the amount of WSA. Though the amount of AMF biomarker NLFA 16:1ω5 was significantly increased by P additions (also the percentage of AMF root colonization was marginally significantly affected), we did not detect a linear correlation of these parameters, also confirmed by stepwise multiple linear regression analyses. This lack of direct correlation corresponds to the result that NLFA 16:1ω5 was only affected by single P additions (statistics not presented), in contrast to the positive effect on WSA which was strongest in the NP treatment. Nevertheless, this parallel increase of NLFA 16:1ω5, intra-radical AMF abundance and WSA following P additions might point toward a partial involvement of AMF in soil aggregation in this system, potentially depending on additional factors (Denef and Six, 2005).

Apart from its impact on soil structure, the overall weak responses to N and P additions of AMF and root abundance is rather unexpected (Treseder, 2004; Johnson, 2010), based on a comparison with previous studies (Gower and Vitousek, 1989;

TABLE 1 | Responses of soil aggregation as well as biotic and abiotic soil factors to N and P additions, as well as the interaction between N and P additions (N:P) (two-way linear mixed-effects models, *F*-values (*P*-values) are presented).

	N	P	N:P
	***df* = 1, 9**	***df* = 1, 9**	***df* = 1, 9**
Water-stable macroaggregates 2013 (%)	0.77 (0.40)	**7.96 (0.02)↑**	1.10 (0.32)
Water-stable macroaggregates 2010 (%)	2.86 (0.13)	**13.29 (0.01)↑**	1.11 (0.32)
Mean weight diameter 2013 (mm)	2.60 (0.14)	1.20 (0.30)	0.03 (0.87)
Mean weight diameter 2010 (mm)	0.01 (0.93)	2.05 (0.19)	0.04 (0.85)
BIOTIC VARIABLES IN 2013			
AMF hyphal length (m cm^{-3} soil)	4.24 (0.07)↓	0.00 (0.97)	1.08 (0.33)
non-AMF hyphal length (m cm^{-3} soil)	4.08 (0.07)↓	3.73 (0.09)↑	0.45 (0.52)
Soil NLFA 16:1ω5 content (nmol cm^{-3} soil)	2.77 (0.13)	**6.49 (0.03)↑**	0.68 (0.43)
AMF root colonization (%)	0.16 (0.70)	3.90 (0.08) ↑	1.81 (0.21)
AMF colonized root length (cm cm^{-3} soil)	0.01 (0.93)	0.82 (0.39)	0.13 (0.73)
Live root length (cm cm^{-3} soil)	0.27 (0.61)	0.27 (0.62)	0.17 (0.69)
ABIOTIC VARIABLES			
C:N ratio	1.96 (0.20)	0.48 (0.51)	0.42 (0.53)
Total P (mg kg^{-1} soil)[1]	0.87 (0.37)	**5.64 (0.04)↑**	0.05 (0.83)
pH[a]	1.85 (0.21)	0.19 (0.67)	0.06 (0.81)
Al (g kg^{-1} soil)[1]	0.03 (0.86)	1.96 (0.20)	1.89 (0.20)
Fe (g kg^{-1} soil)[1]	2.82 (0.13)	0.00 (0.96)	0.89 (0.37)
Ca (mg kg^{-1} soil)[1]	0.02 (0.88)	0.52 (0.49)	4.45 (0.06)

[a] *Data have been measured based on pooled soil samples per plot (n = 4). Bold values indicate significant effects, arrows the respective direction (positive or negative).*

TABLE 2 | Results of simple linear regression analyses of biotic and abiotic predictor variables with the amount of water-stable macroaggregates (based on linear mixed-effects models including *Block* and *Plot* as random effects).

Predictor variable	df	*t*-value	*P*-value
BIOTIC VARIABLES			
AMF hyphal length (m cm^{-3} soil)	31	0.28	0.78
non-AMF hyphal length (m cm^{-3} soil)	31	−0.59	0.56
Soil NLFA 16:1ω5 content (nmol cm^{-3} soil)	31	1.41	0.17
AMF root colonization (%)	31	0.72	0.48
AMF colonized root length (cm cm^{-3} soil)	31	0.94	0.36
Live root length (cm cm^{-3} soil)	31	0.93	0.36
ABIOTIC VARIABLES			
C:N ratio	31	**−2.10**	**0.04**
Total C (mg kg^{-1} soil)	31	1.87	0.07
Total N (mg kg^{-1} soil)	31	**2.49**	**0.02**
Total P (mg kg^{-1} soil)[1]	8	1.81	0.10
pH[a]	8	−0.77	0.46
Al (g kg^{-1} soil)[1]	8	0.27	0.79
Fe (g kg^{-1} soil)[1]	8	−1.37	0.20
Ca (mg kg^{-1} soil)[1]	8	1.03	0.32

[a] *Soil data have been measured based on pooled soil samples per plot (n = 4). Bold values indicate significant correlations.*

Treseder, 2004; Wright et al., 2011). In the case of AMF, the reported increase in extraradical AMF abundance in response to P additions is rather unexpected considering the theory of reduced plant investment toward mycorrhizal structures following nutrient applications (Treseder, 2004; Johnson, 2010). However, there have been several previous reports on increased abundance following P additions, which is mainly discussed in the light of primary nutrient limitation of the fungus itself (Bolan et al., 1984; Olsson et al., 1997; Treseder and Allen, 2002; own unpublished data). Nevertheless, the overall weak response of "nutrient uptake structures" may be related to rather low nutrient inputs compared to other fertilization experiments (Treseder and Vitousek, 2001; Johnson et al., 2003; Wright et al., 2011). Additionally, the abundance of AMF was relatively low with average root colonization values of 25%, potentially indicating a low AMF dependency of plants in this system (Powell et al., 2009; Reinhart et al., 2012).

Though we did not detect (treatment related) correlating factors clearly explaining the observed increase in soil aggregation, the repeated finding of these patterns at different sampling times substantiates the reported treatment effects. Differences in the strength of effect sizes among sampling times might be associated with a strong initial response attenuating in the long-term (Comins and McMurtrie, 1993; Norby et al., 2007), though this may only partly explain differences among control plots. Regarding the experimental design, lower sample sizes in 2010 may have resulted in stronger random deviations causing high effect sizes (Barto and Rillig, 2012). Additionally, different conditions among sampling times may have directly or indirectly affected the amount of WSA in the soil, e.g., regarding rainfall events: February to May 2010 represented considerably drier months than the comparative period in 2013 (Thorsten Peters, personal communication). The rather low effect size in 2013 might be another reason for the inability to detect direct causal agents responsible for the increase in WSA, since also smaller changes may have caused the observed deviations. Nevertheless, in 2010 similarly no correlating factors have been detected despite the inclusion of several biotic and abiotic measurements (the same parameters as presented here were tested; data not shown).

Soil aggregation processes involve complex interactions among biotic and abiotic agents (Jastrow et al., 1998; Six et al., 2004; Barto et al., 2010). Beside the large evidence for the involvement of hyphae and roots in aggregate formation (Rillig and Mummey, 2006; Hallett et al., 2009), other studies previously likewise reported a lack of correlation among these factors (Degens et al., 1994; Eviner and Chapin, 2002; Piotrowski et al., 2004). Additionally, in tropical soils knowledge on soil aggregation processes is scarce, mainly based on agricultural systems (Feller and Beare, 1997; Cardoso and Kuyper, 2006). Within the available literature on tropical soils partly AMF (Nobrega et al., 2001) and also roots (Six et al., 2002) have been mentioned as influential factors. However, especially in highly weathered and acidic soils mainly pH and related Fe and

Al contents, which affect electrostatic interactions and mineral-mineral bonding capacities, seem to have a strong impact (Denef et al., 2002; Six et al., 2002; Denef and Six, 2005). Nevertheless, in our study site different factors seem to play a more predominant role. The significantly positive correlation of WSA with total N values (though the direction of causality remains hidden) might indicate a predominant role of microbial activity in this system (Tripathi et al., 2008), whereas the marginally significant positive relation with total C points toward an involvement of other soil organic matter and C pools (Feller and Beare, 1997; Piccolo and Mbagwu, 1999; Six et al., 2000b). Macrofauna represents another potential candidate, e.g., springtails (Siddiky et al., 2012), but also direct and indirect interactions of plant and microbial communities might play a role (Gransee and Wittenmayer, 2000; Eviner and Chapin, 2002; Piotrowski et al., 2004). Finally, even though we did not detect effects at the level of abundances of biotic factors, it is possible that community composition, and with this community-level trait shifts, were responsible for the observed effects. For example, even though AMF abundance did not explain effects, community composition, likely to change in response to fertilization in this system (Camenzind et al., 2014), might explain it, if for example a shift toward AM fungal phylotypes with increased soil aggregation abilities, determined by species-level trait values (Lehmann and Rillig, 2015; Rillig et al., 2015), occurred.

In conclusion, our data show an increase in WSA following P additions, which is not related to changes in AMF and root abundances, factors which have been previously described as important aggregating agents. This inability to detect but also predict underlying mechanisms of soil aggregation highlights existing knowledge gaps on soil biotic and abiotic interactions in tropical forest soils. Soil structure represents a very important ecosystem component, especially in tropical forests which serve as large terrestrial C pool and are prone to erosion due to high rainfall events. In contrast to N deposition, potential future P depositions due to anthropogenic activities are still under debate: Mahowald et al. (2005, 2008) proposed increasing depositions at least in certain areas including the Andes, which was partly detected also in the study area (Jörg Bendix and Wolfgang Wilcke, personal communication). Furthermore, nutrient availability is affected by increasing land use intensity in the tropics. Therefore, consequences of P additions on soil aggregation processes, which are indicated by the presented data, might have an impact on other ecosystem processes, since soil structure represents such a crucial component of ecosystem functioning.

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Soil N_2O fluxes along an elevation gradient of tropical montane forests under experimental nitrogen and phosphorus addition

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Nutrient deposition to tropical forests is increasing, which could affect soil fluxes of nitrous oxide (N_2O), a powerful greenhouse gas. We assessed the effects of 35–56 months of moderate nitrogen (N) and phosphorus (P) additions on soil N_2O fluxes and net soil N-cycling rates, and quantified the relative contributions of nitrification and denitrification to N_2O fluxes. In 2008, a nutrient manipulation experiment was established along an elevation gradient (1000, 2000, and 3000 m) of montane forests in southern Ecuador. Treatments included control, N, P, and N+P addition (with additions of 50 kg N ha^{-1} yr^{-1} and 10 kg P ha^{-1} yr^{-1}). Nitrous oxide fluxes were measured using static, vented chambers and N cycling was determined using the buried bag method. Measurements showed that denitrification was the main N_2O source at all elevations, but that annual N_2O emissions from control plots were low, and decreased along the elevation gradient (0.57 ± 0.26–0.05 ±0.04 kg N_2O-N ha^{-1} yr^{-1}). We attributed the low fluxes to our sites' conservative soil N cycling as well as gaseous N losses possibly being dominated by N_2. Contrary to the first 21 months of the experiment, N addition did not affect N_2O fluxes during the 35–56 month period, possibly due to low soil moisture contents during this time. With P addition, N_2O fluxes and mineral N concentrations decreased during Months 35–56, presumably because plant P limitations were alleviated, increasing plant N uptake. Nitrogen plus phosphorus addition showed similar trends to N addition, but less pronounced given the counteracting effects of P addition. The combined results from this study (Months 1–21 and 35–56) showed that effects of N and P addition on soil N_2O fluxes were not linear with time of exposure, highlighting the importance of long-term studies.

Keywords: denitrification, nitrification, net nitrogen cycling, nitrous oxide emissions, nitrogen addition, phosphorus addition, ^{15}N tracing

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INTRODUCTION

Nitrous oxide (N_2O) is both a potent greenhouse gas and a dominant ozone-depleting substance (Denman et al., 2007). Soil emissions are the largest natural (i.e., non-anthropogenic) source of N_2O (Ehhalt et al., 2001), which is mainly produced and consumed by the microbial processes of nitrification and denitrification (Chapuis-Lardy et al., 2007). While nitrification is an obligate

aerobic process, which depends on ammonium (NH_4^+) and/or organic nitrogen (N) as substrates, denitrification is an anoxic process which is controlled mainly by the soil aeration/oxygen status (i.e., soil water content), nitrate (NO_3^-) availability, microbially-available organic carbon, and soil pH (Firestone and Davidson, 1989). Thus, soil N availability and water content play a crucial role in controlling the amounts and relative ratios of N-oxide fluxes from soils.

Given the controlling factors for N_2O flux, moist tropical forests with high natural N availability have the potential to be strong N_2O sources. Indeed, tropical forest soils currently account for 30% of global N_2O emissions from unmanaged soils (Ehhalt et al., 2001). However, since atmospheric N deposition from biomass burning, fertilizer use, and industrialization is rapidly increasing (Hietz et al., 2011), it is possible that N_2O emissions in tropical forests may increase (Koehler et al., 2009; Martinson et al., 2013). In temperate forest ecosystems, elevated N deposition has been shown to increase soil N availability and accelerate soil N cycling, causing substantial N losses in the form of N_2O emissions (e.g., Butterbach-Bahl et al., 1998; Gundersen et al., 1998). In a meta-analysis of N enrichment effects on greenhouse gas fluxes, Liu and Graever (2009) showed that the N_2O response to elevated N input could even be stronger in tropical forests than in other ecosystems. These authors suggested that the strength of the tropical forest response to N enrichment may be due to the phosphorus (P) limitation (rather than N limitation) of many tropical soils.

Tropical montane forests (TMFs) make up over 11% of the world's tropical forests. They occur across large elevation gradients and contain a large variety of environmental conditions (FAO, 1993; Bubb et al., 2004). TMFs usually exhibit "conservative" soil N cycling (similar rates of mineral N production and consumption), which results in low N losses. This is in contrast to tropical lowland forest soils, which typically have a more "leaky" soil N cycle; they often have relatively high soil N cycling rates (Vitousek and Matson, 1988; Corre et al., 2010), N-oxide (NO, N_2O) fluxes (e.g., Matson and Vitousek, 1987; Keller et al., 2005; Purbopuspito et al., 2006; Koehler et al., 2009), and NO_3^- leaching (e.g., Hedin et al., 2003; Dechert et al., 2005; Schwendenmann and Veldkamp, 2005). However, N addition experiments in TMFs of Hawaii (Hall and Matson, 2003) and Panama (Koehler et al., 2009; Corre et al., 2014) have shown increases in soil mineral N production - especially nitrification rates - and N-oxide fluxes in as little as 1–2 years after the onset of N addition. In the Panamanian TMF, soil N_2O emissions during the third and fourth year of N addition reached levels as high as those from the lowland forest, which had already experienced 11–12 years of N addition (Corre et al., 2014). Therefore, there is a strong potential for TMF soil to become a significant N_2O source when subjected to chronic N input.

Tropical South America is also experiencing increases in atmospheric P deposition, mainly due to biomass burning (Mahowald et al., 2005). As mentioned above, soil P limitation may affect the response of soil N cycling (and therefore N_2O flux) to N enrichment (Liu and Graever, 2009). Hall and Matson (1999), found that N addition to a Hawaiian tropical forest soil resulted in higher inorganic N concentrations and N_2O emissions in P-limited soils as compared to N-limited soils. However, field studies with P addition have shown varying results. In a plantation of N-fixing trees in Indonesia, a one-time application of 100 kg P ha^{-1} followed by 2 years of measurements, exhibited decreased soil N contents and N_2O emissions, with increased plant N uptake (Mori et al., 2013). In contrast, Wang et al. (2014) showed that the interaction between N and P addition could result in increased N_2O emissions in a tropical lowland forest in Southern China. Phosphorus is known to limit plant growth in TMFs (Tanner et al., 1998; Homeier et al., 2012), but the effect of elevated P deposition on soil N cycling in these areas has been little studied.

Since responses of ecosystem processes to chronic nutrient addition may be non-linear with time (e.g., Aber et al., 1998), quantifying changes in N-oxide fluxes and their controlling factors need to be conducted in long-term and large-scale nutrient manipulation experiments. Here, we report the changes in soil N_2O fluxes, contributions of nitrification and denitrification to N_2O fluxes, and net rates of soil N cycling (an index of plant-available N) during Months 35–56 of moderate N, P, and combined N+P addition (with additions of 50 kg N ha^{-1} yr^{-1} and 10 kg P ha^{-1} yr^{-1}), along an elevation gradient (1000, 2000, and 3000 m) of TMFs in southern Ecuador.

Martinson et al. (2013) reported the effects of nutrient manipulation on soil N_2O fluxes from Months 1–21 of this experiment. Along the elevation gradient, they observed higher soil N_2O emissions from soils with N and N+P addition, with soils at 1000 m responding more rapidly than the higher elevations. They also observed slight increases in net nitrification at 2000 and 3000 m (as compared to the previously-undetectable net nitrification activity) with N and N+P additions. However, they did not detect any effect of P addition on soil N_2O fluxes or net soil N cycling rates at any elevation.

The objectives of the present study were to (1) determine the cumulative effect of 35–56 months of moderate nutrient additions on soil N_2O fluxes and net soil N cycling rates, and (2) assess the contributions of nitrification and denitrification to soil N_2O fluxes. By using an elevation gradient, we were also able to assess whether N_2O fluxes and/or the microbial processes responsible for N_2O production changed with the different environmental conditions associated with elevation (Supplementary Table 1). In response to nutrient addition, we hypothesized that soil N_2O fluxes, together with net soil N cycling rates, would further increase as N and N+P additions continued, whereas the moderate P addition might begin to alleviate P limitation, enabling more N uptake and therefore decreasing soil N_2O emissions.

MATERIALS AND METHODS

Study Area

The study area was located in the Andes of southern Ecuador, in the provinces of Loja and Zamora Chinchipe. The experiment

Abbreviations: HIP, Hole-in-the-pipe; IRMS, Isotope ratio mass spectrometry; TMF, Tropical montane forest; WFPS, Water-filled pore space.

was conducted within and close to Podocarpus National Park, in three old-growth forest sites spanning an elevation gradient from 1000 to 3000 m above sea level (Supplementary Table 1; Homeier et al., 2012; Martinson et al., 2013). Forest types across the elevation gradient ranged from premontane at "1000 m" (990–1100 m), to lower montane at "2000 m" (1950–2100 m) and upper montane at "3000 m" (2900–3050 m) (Homeier et al., 2012). At 1000 m, sandy Cambisol soil (covered by only a thin layer of decomposing leaves) had developed from deeply weathered granitic rock (Litherland et al., 1994). At 2000 and 3000 m, loamy textured Cambisol and Histosol soil, respectively, had developed from metamorphic schists (Litherland et al., 1994); these soils were covered by 10–40 cm of thick organic layers (Supplementary Table 1). The study area displayed only slight seasonal variability (Emck, 2007). Mean annual air temperature decreased with elevation from 19.4°C at 1000 m, 15.4°C at 2000 m to 9.4°C at 3000 m. Mean annual precipitation ranged from 1950 mm yr^{-1} (2000 m) to 4500 mm yr^{-1} (3000 m), with intermediate rainfall of 2230 mm yr^{-1} at 1000 m (Moser et al., 2007). Ambient bulk and dry deposition of N and P in the study region ranged between 14 and 45 kg N ha^{-1} yr^{-1} and 0.4 and 4.9 kg P ha^{-1} yr^{-1}, with an increasing tendency for deposition from 1998 to 2010 (Boy et al., 2008; Homeier et al., 2012) and thereafter (personal communication, W. Wilcke). Due to data gaps, we were not able to get detailed information on monthly rainfall during our measurement period. We attribute differences between the three forest sites to the combination of climatic, vegetation, and soil factors associated with the different elevations. However, the elevations themselves were not replicated, so our results may not represent these elevations in other TMF areas.

Experimental Design

At each elevation, we established nutrient manipulation experiments (NUMEX) with 16 plots (20 × 20 m each) equally distributed to four blocks. The four blocks served as replicates and included minimal topographic differences (50–100 m) within each elevation. Each block consisted of four plots: N, P, and N+P additions, and untreated controls; these plots were separated by at least 10 m (Homeier et al., 2013; Martinson et al., 2013). Fertilization started in 2008 with two equal applications per year (February/March and August/September), with the exception of 2010 when there was a 4-month delay of the second fertilization. Fertilizers were applied in solid form at rates of 50 kg N ha^{-1} yr^{-1} of urea (CH_4N_2O) and 10 kg P ha^{-1} yr^{-1} of sodium hydrogen phosphate ($NaH_2PO_4 \cdot H_2O$ and $NaH_2PO_4 \cdot 2H_2O$, with analytic grade quality).

Soil N_2O Flux, Temperature, Moisture and Mineral N Concentrations

Measurements of soil N_2O flux, temperature, moisture, and mineral N concentrations followed the same procedure described in detail by Martinson et al. (2013). Measurements were performed monthly from Month 35 to Month 56 of the experiment (with January 2008 defined as Month 1) in three out of the four blocks, with a minimum distance of 2 m to plot borders for the nutrient-addition plots. In each plot, measurements were conducted at four locations that were laid out in a stratified random pattern (Martinson et al., 2013); after Month 37, we added one additional location per plot as part of a small scale manipulation study, to measure soil trace gas fluxes. Since we were interested in long-term effects of nutrient deposition rather than the transitory peaks of N_2O that occur after N addition, we only included measurements that were taken at least 3 weeks after fertilization. This timespan was chosen based on a study from our working group in Panama, where mineral N concentrations and N_2O emissions peaked within 3 weeks following N application in a TMF (Koehler et al., 2009). As shown by Wullaert et al. (2010), we could assume that minimal leaching occurred during those 3 weeks; therefore, the N_2O measured throughout the rest of the year can be considered a realistic long-term response to increasing soil nutrients.

Soil N_2O fluxes were measured using static vented chambers with permanently installed round polyvinyl chloride chamber bases (area 0.04 m^2, height 0.15 m, ∼0.03 m inserted into the soil) and polyethylene chamber hoods with a Luer lock sampling port and a vent for pressure equilibrium (0.16 m height of chamber cover, 0.03 m overlapping width with chamber base for tight cover, and 12 L total volume). Gas samples were taken at 2, 14, 26, and 38 min or at 3, 13, 23, and 33 min after chamber closure and stored in pre-evacuated glass containers (60 ml vials until Month 40 and 12 ml Exetainers® afterwards). Gas samples were either analyzed in Ecuador or in Germany, after shipping as over-pressured samples in Labco Exetainers® (Labco Limited, UK). We have tested these Exetainers® for their quality during extended sample storage and aircraft transport (see also Glatzel and Well, 2008). Gas samples were analyzed using gas chromatographs (Shimadzu GC-14B, Duisburg, Germany for samples analyzed in Ecuador and GC 6000 Vega Series 2, Carlo Erba Instruments, Milan, Italy for samples analyzed in Germany; both of these and the standard gases are owned by our group and were calibrated regularly) equipped with an electron capture detector and autosamplers. Nitrous oxide concentrations were determined from the comparison of integrated peak areas from samples to three or four standard gases (ranging from 350 to 3000 ppb; Deuste Steininger GmbH, Mühlhausen, Germany). Fluxes of N_2O, expressed as N_2O-N flux per area (μg N m^{-2} h^{-1}), were calculated from the linear increase of N_2O concentration in the chamber headspace over time, corrected for the air pressure and temperature measured at the time of field sampling. Annual soil N_2O fluxes were approximated by applying the trapezoid rule on time intervals between measured flux rates. Annual rates combine measurements from Months 1–21 (using data from Martinson et al., 2013) to Months 35–56 (this study), assuming constant flux rates per day.

Soil temperature was measured parallel to gas sampling at a 0.05-m depth close to each of the four chamber bases per plot using a GTH 175/Pt-E digital precision thermometer (Greisinger electronics GmbH, Regenstauf, Germany). Soil moisture and mineral N concentrations were determined parallel to gas sampling for each plot from pooled soil samples of the top 5 cm of soil, consisting of four samples taken within 1 m of each chamber. Soil moisture was determined by oven-drying subsamples at 105° C for at least 24 h and was expressed as percentage of water-filled pore space (WFPS) using the

measured bulk densities in the top 5 cm of soil (reported by Martinson et al., 2013) and particle densities of 2.65 g cm^{-3} for mineral soil at 1000 m and 1.4 g cm^{-3} for organic layers at 2000 and 3000 m (Linn and Doran, 1984; Breuer et al., 2002). Soil extraction for mineral N concentration determination was carried out *in-situ* in order to avoid alterations of mineral N concentrations due to storage after field sampling (Arnold et al., 2008). A subsample of soil was added into a prepared extraction bottle with 150 ml of 0.5 mol l^{-1} K_2SO_4 solution. After returning to the laboratory on the same day, samples were shaken (1 h), filtered and kept frozen until lab analyses were conducted. Analysis of mineral NH_4^+ and NO_3^- concentration was done at the University of Goettingen, using continuous flow injection colorimetry (Cenco/Skalar Instruments, Breda, Netherlands); NH_4^+ was analyzed by the Berthelot reaction method (Skalar Method 155-000) and NO_3^- by the copper-cadmium reduction method with NH_4Cl buffer but without ethylenediamine tetraacetic acid (Skalar Method 461-000).

Net Soil N Cycling Rates: Ammonification and Nitrification

Net rates of soil N cycling were determined three times, in Months 38 and 48 (just over 1 month and about 4 months following fertilization) and in Month 52 (about 2 months following fertilization), using the *in-situ* buried bag method (Hart et al., 1994). Two pairs of intact soil cores were taken from the top 5 cm of mineral soil (at 1000 m) or organic layer (at 2000 and 3000 m) in each plot of all four blocks. One soil core of each pair was extracted immediately in the field with 0.5 mol L^{-1} K_2SO_4 (as described above); the other soil core was put in a plastic bag, inserted back into the soil to incubate for 10 days and afterwards extracted. Net soil N cycling rates of each sampling pair were calculated by subtracting the initial soil mineral N concentrations from mineral N concentrations of incubated soils. Net ammonification is the difference in NH_4^+ concentrations and net nitrification is the difference in the NO_3^- concentrations.

^{15}N Tracing to $^{15}N_2O$: Contributions of Nitrification and Denitrification to Soil N_2O Flux

Short-term tracing from $^{15}NH_4^+$ or $^{15}NO_3^-$ to $^{15}N_2O$ was used to determine the contributions of nitrification and denitrification to soil N_2O fluxes; we used the same method applied in a montane forest in Panama (Corre et al., 2014). Tracing was conducted in all four replicate plots of the control and N-addition treatments at all three elevations and was carried out in Month 43 and Month 49, 4, and 5 months after the last fertilization. In each of the four control or N-addition plots, two additional chamber bases (same dimensions and material as described above) were installed >10 m apart, at least 3 weeks prior to sampling. In N-addition plots, the bases were >2 m from plot edges. For the second sampling, the chamber bases were located close to the previous chambers of the same ^{15}N source but always upslope to prevent any possible influences from previously applied ^{15}N. Each of the two chambers in a plot was labeled separately with either $^{15}NO_3^-$ or $^{15}NH_4^+$.

The amounts of added ^{15}N (either NH_4^+ or NO_3^-) were calculated based on the extant soil mineral N levels in the control and N-addition plots such that the added ^{15}N would be at most 50% of the native levels and the volume of solution would not increase the soil moisture contents. In each plot, 0.52 mg ^{15}N-KNO_3 in 50 ml distilled water was applied to the soil within one chamber (0.04 m^2 area) and 13.29 mg of ^{15}N-$(NH_4)_2SO_4$ in 50 ml distilled water was applied to the soil within the other chamber. Half of the amount of the ^{15}N solution was injected about 25 cm deep with a side-port needle at several points inside the chamber. The other half was sprayed with a hand sprayer onto the surface of the soil after removal of the leaf litter layer (which was returned afterwards). Transparent plastic covers (0.9 × 0.6 m) were put 0.5 m above the chamber bases 1–2 days before labeling to prevent immediate leaching losses of the applied ^{15}N tracers in case of rainfall. These applied amounts of $^{15}NO_3^-$ and $^{15}NH_4^+$ were the same for all plots and represented 3–30% and 7–46% of the native NO_3^- and NH_4^+ levels, respectively, in the top 5 cm of soil across all control and N-addition plots.

Thirty minutes after ^{15}N application, gas samples were taken with a syringe at 2, 17, and 32 min following chamber closure and stored as overpressured samples in 100 ml pre-evacuated glass vials with butyl rubber septa. These glass vials were tested as leak proof in an earlier study (Corre et al., 2014). Gas samples were analyzed for N_2O concentrations using the same gas chromatograph described above and $^{15}N_2O$ was determined using isotope ratio mass spectrometry (IRMS; Finnigan Delta^{Plus} XP, Thermo Electron Corporation, Bremen, Germany). Following the third of the three gas samples per chamber, we took a soil sample of the top 5 cm in the center of each chamber base to determine soil moisture and mineral N concentrations following the procedures described above. Additionally, ^{15}N from NH_4^+ and NO_3^- was determined by the ^{15}N diffusion procedures described in detail by Corre and Lamersdorf (2004) and analyzed using IRMS (Delta C, Finnigan MAT, Bremen, Germany). Contributions of nitrification and denitrification to soil N_2O fluxes were calculated following the same calculations given by Corre et al. (2014).

Statistical Analysis

Data were checked for normality and homoscedasticity, and we used either square root or logarithmic transformation (adding a constant value if the dataset included negative values) for data with non-normal distribution and unequal variance. If after transformation the data were still non-normally distributed we used non-parametric statistical tests.

The influence of soil factors (moisture, temperature, mineral N concentrations) on soil N_2O fluxes was assessed using Pearson's correlation tests: first, across the elevation gradient considering the control plots only to assess which of these soil factors control N_2O fluxes under ambient nutrient conditions, and second for each elevation considering all treatment plots to determine if changes in these soil factors due to nutrient amendment influence changes in N_2O fluxes. These analyses were conducted for the measurements from Month 35–56 on the treatment means (average of three replicate plots) on each sampling day.

Effects of elevation and nutrient addition on time series data (soil N_2O flux, temperature, WFPS, mineral N concentration and net N-cycling rates) were assessed using linear mixed effects (LME) models (Piepho et al., 2004; Crawley, 2012). Analyses were based on plot means (the average of four or five chamber measurements for N_2O and two measurements for net soil N cycling) with three replicate plots (for all parameters) or four replicate plots (for soil N-cycling rates). Elevation effects were assessed for control plots only and nutrient-addition effects were assessed separately for each of the three elevations. Elevation or treatments were considered fixed effects whereas sampling month and plot (as spatial replication) were included as random effects. The following structures were included in the LME model if this improved the relative goodness of the model fit based on the Akaike information criterion: (1) a first-order temporal autoregressive process accounting for decreasing correlation of measurements with increasing time difference (Zuur et al., 2009) and (2) a variance function varIdent to model heteroscedasticity of residual variances (Crawley, 2012). The significance of the fixed effects was then determined by analysis of variance and stepwise model simplification.

For the short-term ^{15}N tracing method of N_2O sources, we first assessed the effects of added ^{15}N solution on soil parameters (mineral N concentrations, WFPS, NO_3^-/NH_4^+ ratio) and soil N_2O fluxes for each measurement campaign, elevation and treatment. We compared $^{15}NH_4^+$- with $^{15}NO_3^-$-labeled chambers and both with reference (without ^{15}N) chambers that were measured in the nearest sampling months, using Paired *T*-tests. Second, we tested the differences in relative contributions of nitrification and denitrification to N_2O fluxes between years for each elevation and treatment, between elevations for the control plots only and between treatments for each elevation, using either *T*-tests (independent and paired) or a Mann–Whitney *U*-test.

The significance level was defined at $P \leq 0.05$ and mean values in the text are given with ± standard error (SE). Statistical analyses were conducted using R 2.14.0 (R Development Core Team, 2012).

RESULTS

Effects of Elevation on Soil Temperature, Wfps, and Net N Cycling under Ambient Environmental Conditions

From Month 35 to 56, soil temperature and WFPS in the top 5 cm of soil in control plots differed between elevations (**Table 1**) but showed no clear seasonal pattern at any elevation. Soil temperature decreased with increasing elevation ($P < 0.001$), while WFPS was highest at 2000 m, followed by 3000 m and then 1000 m ($P < 0.001$).

Net ammonification rates in control plots did not differ across the elevation gradient ($P = 0.126$; **Table 2**), which was caused by the large spatial variability (i.e., large SE), but net nitrification rates were larger at 1000 m than at 2000 and 3000 m ($P < 0.001$; **Table 2**). At all elevations, the dominant form of mineral N in the top 5 cm of soil was NH_4^+ (**Table 2**). Soil NH_4^+ was higher at 1000 and 2000 m compared to 3000 m ($P < 0.001$), and did not vary markedly between the measurement periods. Similar to net nitrification, NO_3^- decreased with increasing elevation ($P < 0.001$; **Table 2**), and larger NO_3^- concentrations at 1000 m

TABLE 1 | Mean (±SE, $n = 3$) soil temperature, water-filled pore space (WFPS) and N_2O fluxes in tropical montane forests along a 1000-m to 3000-m elevation gradient.

Elevation (m)	Treatment	Soil temperature (°C)	WFPS (%)	Soil N_2O fluxes (hourly) from Months 35–56 ($\mu g\ N\ m^{-2}\ h^{-1}$)	Soil N_2O fluxes (annual) from Months 35–56 ($kg\ N\ ha^{-1}\ yr^{-1}$)	Soil N_2O fluxes (annual) from Months 1–56 ($kg\ N\ ha^{-1}\ yr^{-1}$)
1000	Control	18.43 ± 0.10^{A}	43.35 ± 3.87^{C}	$6.40 \pm 3.17^{A,ab}$	0.57 ± 0.26	0.50 ± 0.16
	Nitrogen (N)	18.50 ± 0.04	45.26 ± 6.91	7.50 ± 1.20^{a}	0.64 ± 0.08	0.71 ± 0.17
	Phosphorus (P)	18.60 ± 0.09	51.41 ± 9.69	3.63 ± 1.31^{b}	0.33 ± 0.13	0.43 ± 0.02
	N + P	18.66 ± 0.07	54.06 ± 6.68	6.73 ± 0.67^{a}	0.59 ± 0.06	0.92 ± 0.15
2000	Control	14.67 ± 0.28^{B}	71.12 ± 4.21^{A}	$2.05 \pm 0.64^{B,ab}$	0.17 ± 0.06	0.16 ± 0.09
	N	14.77 ± 0.19	71.64 ± 2.00	2.99 ± 0.42^{a}	0.27 ± 0.04	0.31 ± 0.04
	P	14.81 ± 0.17	72.88 ± 2.41	1.09 ± 0.40^{b}	0.09 ± 0.03	0.09 ± 0.06
	N + P	14.64 ± 0.18	69.00 ± 1.76	2.98 ± 0.51^{a}	0.25 ± 0.05	0.48 ± 0.02
3000	Control	$9.80 \pm 0.26^{C,b}$	$58.58 \pm 0.82^{B,a}$	0.47 ± 0.62^{B}	0.05 ± 0.04	-0.08 ± 0.01
	N	9.61 ± 0.26^{b}	57.00 ± 2.25^{a}	1.00 ± 0.21	0.10 ± 0.02	0.20 ± 0.04
	P	10.03 ± 0.26^{a}	50.34 ± 2.13^{ab}	0.78 ± 0.75	0.07 ± 0.05	-0.02 ± 0.13
	N + P	9.74 ± 0.10^{b}	44.61 ± 6.91^{b}	1.40 ± 0.33	0.12 ± 0.02	0.26 ± 0.07

Measurements of temperature, WFPS and hourly N_2O flux occurred between November 2010 and August 2012 (Months 35–56 of nutrient addition). Values given for annual N_2O fluxes also show the combined measurements from Months 1 to 21 (Martinson et al., 2013) and Months 35 to 56 (this study). For soil temperature, WFPS and hourly soil N_2O fluxes, means followed by different capital letters indicate significant differences across the elevation gradient for the control plots, and means followed by small letters indicate significant differences among treatments within each elevation (linear mixed effects model at $P \leq 0.05$). Annual soil N_2O fluxes were approximated by applying the trapezoid rule on time intervals between measured flux rates, assuming constant flux rates per day (Note: hourly and annual fluxes do not include any transitory peaks that may have occurred directly following nutrient addition, as measurements always occurred at least 3 weeks after fertilization).

TABLE 2 | Mean (±SE) net soil N cycling rates ($n = 4$) and soil mineral N concentrations ($n = 3$) in the top 5 cm of tropical montane forest soils along a 1000-m to 3000-m elevation gradient.

Elevation (m)	Treatment	Net soil N cycling rates (mg N m^{-2} d^{-1})		Soil mineral N concentration (mg N m^{-2})	
		Ammonification	Nitrification	NH_4^+	NO_3^-
1000	Control	$15.24 \pm 5.59^{A,a}$	35.48 ± 6.82^{A}	335.79 ± 31.38^{A}	$43.41 \pm 26.01^{A,c}$
	Nitrogen (N)	3.02 ± 1.73^{ab}	42.93 ± 1.83	308.96 ± 25.59	76.07 ± 15.40^{a}
	Phosphorus (P)	19.71 ± 8.45^{a}	52.95 ± 7.96	317.02 ± 15.62	19.44 ± 8.33^{d}
	N+P	-3.76 ± 2.23^{b}	38.56 ± 3.28	328.13 ± 55.86	58.60 ± 7.88^{b}
2000	Control	$1.25 \pm 5.64^{A,b}$	$0.25 \pm 0.29^{B,b}$	$359.96 \pm 17.12^{A,c}$	$9.59 \pm 2.33^{B,b}$
	N	44.97 ± 19.37^{a}	4.39 ± 3.02^{a}	745.60 ± 49.82^{a}	44.08 ± 14.81^{a}
	P	1.58 ± 4.12^{b}	0.34 ± 0.30^{b}	324.51 ± 20.85^{c}	8.78 ± 2.33^{b}
	N+P	19.04 ± 6.97^{a}	8.39 ± 2.45^{a}	563.17 ± 57.76^{b}	60.49 ± 6.46^{a}
3000	Control	$-0.04 \pm 1.93^{A,c}$	$-0.03 \pm 0.09^{B,b}$	$217.95 \pm 10.71^{B,b}$	$3.77 \pm 0.83^{C,c}$
	N	21.31 ± 5.48^{a}	1.24 ± 0.71^{a}	504.82 ± 99.84^{a}	22.25 ± 1.11^{a}
	P	5.69 ± 3.79^{bc}	0.08 ± 0.11^{b}	159.51 ± 9.94^{c}	2.53 ± 0.77^{d}
	N+P	14.57 ± 5.83^{ab}	1.76 ± 1.36^{a}	248.07 ± 64.80^{b}	6.77 ± 1.36^{b}

Soil mineral N concentrations were measured monthly from Months 35 to 56 of nutrient addition. Net N-cycling rates were measured three times (Months 38, 48, and 52 of nutrient addition). Means followed by different capital letters indicate significant differences across the elevation gradient for the control plots and means with superscript small letters indicate significant differences among treatments for each elevation (linear mixed effects model at $P \leq 0.05$).

during Month 38, Months 47–48, and Months 55–56, and at 2000 m during Month 47, coincided with months of large litterfall (Homeier et al., unpublished data on litterfall).

Effects of Elevation on Soil N_2O Fluxes under Ambient Environmental Conditions

Soil N_2O fluxes in control plots decreased with increasing elevation; hourly fluxes at 1000 m were more than three times larger than at 2000 m and more than 13 times larger than at 3000 m (**Table 1**). Temporal variability of N_2O fluxes from the control plots was largest at 1000 m, but there was no clear seasonal trend (**Figure 1**).

In the control plots at each elevation, addition of ^{15}N solutions for the short-term ^{15}N tracing of N_2O did not affect soil N_2O fluxes, WFPS or mineral N concentrations ($P \geq 0.060$) as compared to the reference (without ^{15}N) chambers, except at 2000 m during the first measurement campaign, where addition of $^{15}NH_4^+$ solution increased soil NH_4^+ concentrations ($P < 0.009$). The relative contributions of nitrification and denitrification to N_2O fluxes did not differ between the two measurement campaigns ($P \geq 0.500$), and hence we report the means (±SE, $n = 4$) of these two periods. Denitrification dominated N_2O fluxes in control plots along the elevation gradient with contributions of 67 ± 26% at 1000 m, 100 ± 0% at 2000 m and 98 ± 3% at 3000 m (**Figure 2**). There was a larger contribution of nitrification to N_2O fluxes at 1000 m than at 2000 m ($P = 0.029$). The amounts of $^{15}N_2O$ emitted during 30 min of chamber closure were very small: up to 0.003% of soil $^{15}NH_4^+$ and up to 0.755% of soil $^{15}NO_3^-$ in the top 5 cm of soil across the elevation gradient.

Across the elevation gradient, soil N_2O fluxes from control plots were positively correlated with soil temperature and NO_3^- (**Table 3**). Soil temperature and soil NO_3^- concentration were negatively correlated with WFPS. Soil temperature was positively correlated with soil NH_4^+ and NO_3^- concentrations. Soil NO_3^- and NH_4^+ concentrations were also positively correlated. Correlation tests performed for each elevation revealed significant correlations at 1000 m only: positive correlations between soil N_2O fluxes and WFPS ($r^2 = 0.59$; $P = 0.004$; $n = 22$) and between soil temperature and NH_4^+ concentrations ($r^2 = 0.52$; $P = 0.013$; $n = 22$).

Effects of Nutrient Addition on Soil Temperature, WFPS, and Net N Cycling Across an Elevation Gradient

Soil temperature and WFPS measured between Month 35 and 56 differed between treatments only at 3000 m ($P < 0.001$; **Table 1**). Soil temperature was higher in P plots compared to all other treatments ($P = 0.006$–0.033) whereas WFPS was lower in N+P plots compared to control ($P = 0.013$) and N plots ($P = 0.026$).

Soil mineral N concentrations measured monthly between Month 35 and 56 were also influenced by nutrient addition (**Table 2**). At 1000 m, NH_4^+ concentrations did not differ between treatments ($P = 0.601$) whereas NO_3^- concentrations decreased in the order of N, N+P, control, and P plots ($P \leq 0.017$; **Table 2**). At 2000 m, NH_4^+ concentrations in N and N+P plots were larger than in control plots ($P < 0.005$) with concentrations in N plots being larger than in N+P plots ($P = 0.007$). Soil NO_3^- concentrations displayed similar differences between treatments as that of NH_4^+ (**Table 2**). At 3000 m, NH_4^+ concentrations were higher in N plots and lower in P plots compared to control and N+P plots ($P < 0.001$) whereas NO_3^- concentrations displayed the same treatment differences described for 1000 m with descending concentrations in the order of N, N+P, control, and P plots ($P < 0.001$; **Table 2**).

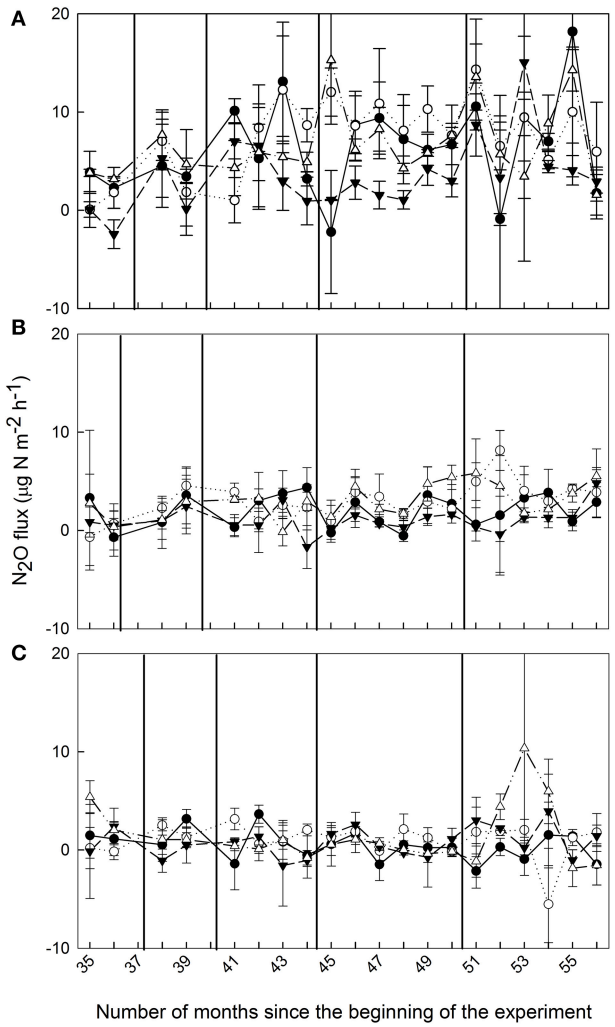

FIGURE 1 | Mean (±SE, $n = 3$) soil N_2O fluxes (μg N m^{-2} h^{-1}) from montane forests at (A) 1000 m, (B) 2000 m, and (C) 3000 m during Months 35–56 of nutrient manipulation: control (*filled circle*), N addition (*open circle*), P addition (*filled triangle*), and N+P addition (*open triangle*). Vertical lines indicate fertilization events.

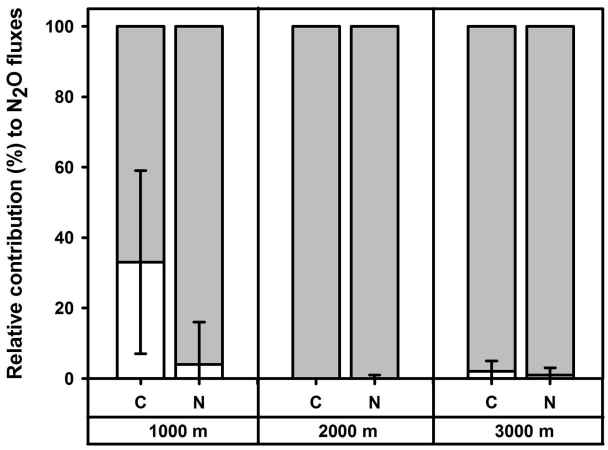

FIGURE 2 | Mean (±SE, $n = 4$) relative contribution (%) of nitrification (*white*) and denitrification (*shaded*) to soil N_2O fluxes in the top 5 cm of forest soils along an elevation gradient, measured in Month 43 and 49 of experimental nutrient addition.

Nutrient addition affected net soil N cycling rates across all elevations (**Table 2**). At 1000 m, net ammonification rates decreased in N+P plots ($P = 0.017$) compared to control and P plots ($P = 0.004$) whereas the N plots showed intermediate rates (**Table 2**). There was no treatment difference detected for net nitrification rates ($P = 0.357$). At 2000 m, net ammonification and nitrification rates increased in N and N+P plots compared to control and P plots ($P = 0.001$–0.033; **Table 2**). At 3000 m, net ammonification rates increased in N ($P = 0.001$) and N+P plots ($P = 0.007$) compared to control plots, and P plots did not differ from the control ($P = 0.196$; **Table 2**). Furthermore, net ammonification rates in N plots were higher than in P plots ($P = 0.029$). Net nitrification rates increased in N ($P = 0.011$) and N+P plots ($P = 0.005$) whereas P plots were comparable with the control ($P = 0.536$).

Effects of Nutrient Addition on Soil N_2O Fluxes Across an Elevation Gradient

At 1000 m, over the entire measurement period, soil N_2O fluxes from nutrient-amended plots were not different from control plots ($P = 0.059$–0.146; **Figure 1A**, **Table 1**). The P plots, however, had lower soil N_2O fluxes compared to N ($P = 0.001$) and N+P plots ($P = 0.004$) (**Table 1**). At 2000 m, nutrient additions had the same effects as at 1000 m; N_2O fluxes from nutrient-amended plots were not significantly different than control plots ($P = 0.119$–0.128), but P plots had lower soil N_2O fluxes compared to N and N+P plots (both $P = 0.002$; **Figure 1B**, **Table 1**). At 3000 m, there were no detectable treatment differences in soil N_2O fluxes ($P = 0.391$–0.651; **Figure 1C**, **Table 1**).

For the short-term ^{15}N tracing method of N_2O sources, addition of ^{15}N solutions did not affect soil N_2O fluxes, WFPS or mineral N concentrations ($P \geq 0.062$) as compared to the reference chambers in each N plot at each elevation. Relative contributions of nitrification and denitrification to soil N_2O fluxes did not differ between the two measurement campaigns for each treatment ($P \geq 0.500$) and hence we reported the average values of these two measurements. We did not detect a significant difference in the sources of N_2O fluxes between control and N plots at any elevation ($P = 0.625$) and mean (± SE, $n = 4$) contributions of denitrification to N_2O fluxes in N plots were 96 ± 12% at 1000 m, 100 ± 1% at 2000 m and 99 ± 2% at 3000 m (**Figure 2**). The amounts of $^{15}N_2O$ emitted during 30 min of chamber closure were maximally 0.004% of soil $^{15}NH_4^+$ and 0.065% of soil $^{15}NO_3^-$ in the top 5 cm across the elevation gradient.

Across all treatments plots for each elevation, correlations of soil N_2O fluxes with soil temperature, WFPS and mineral N varied (**Table 4**). At 1000 m, N_2O fluxes were positively correlated with WFPS. At 2000 m, there was a positive correlation of N_2O fluxes with WFPS, NH_4^+ and NO_3^- concentrations. At

TABLE 3 | Pearson correlation coefficients for monthly average ($n = 66$) soil N_2O flux ($\mu g\ N\ m^{-2}\ h^{-1}$), soil temperature (°C), water-filled pore space (WFPS; %), and mineral N concentrations ($mg\ N\ m^{-2}$) in control plots of forests across an elevation gradient.

	Soil temperature	WFPS	NH_4^+	NO_3^-
Soil N_2O flux	0.51*	−0.13	0.21	0.36*
Soil temperature		−0.40*	0.50*	0.67*
WFPS			0.05	−0.41*
NH_4^+				0.34*

* $P < 0.01$.

TABLE 4 | Pearson correlation coefficients for monthly average ($n = 80$) soil N_2O flux ($\mu g\ N\ m^{-2}\ h^{-1}$), soil temperature (°C), water-filled pore space (WFPS; %), and mineral N concentrations ($mg\ N\ m^{-2}$) of all treatment plots of forests along an elevation gradient.

Elevation (m)		Soil temperature	WFPS	NH_4^+	NO_3^-
1000	Soil N_2O flux	−0.21	0.25*	0.02	0.12
	Soil temperature		−0.29**	0.49**	0.15
	WFPS			−0.16	−0.24**
	NH_4^+				0.36**
2000	Soil N_2O flux	0.09	0.32**	0.21*	0.10**
	Soil temperature		−0.16	0.03	−0.04
	WFPS			0.01	−0.10
	NH_4^+				0.58**
3000	Soil N_2O flux	0.10	−0.04	0.09	0.03
	Soil temperature		−0.01	0.07	0.03
	WFPS			0.33**	0.08
	NH_4^+				0.56**

*$P < 0.05$, **$P < 0.01$.

3000 m, we did not detect any significant correlation between N_2O fluxes and these controlling soil factors.

DISCUSSION

Effects of Elevation on Soil N_2O Fluxes and Controlling Factors under Ambient Environmental Conditions

Annual soil N_2O fluxes from control plots across the elevation gradient (**Table 1**) were lower than those from other TMFs at comparable elevations that reported *in-situ*, year-round measurements, e.g., in Indonesia (0.29–1.11 kg N_2O-N ha^{-1} yr^{-1}; Purbopuspito et al., 2006), Panama (1.13 kg N_2O-N ha^{-1} yr^{-1}; Koehler et al., 2009), Brazil (0.96 kg N_2O-N ha^{-1} yr^{-1}; Sousa Neto et al., 2011), and Peru (0.44–2.23 kg N_2O-N ha^{-1} yr^{-1}; Teh et al., 2014). Our hourly N_2O fluxes (**Table 1**) were, however, within the range of others reported from our general study area (Wolf et al., 2011) as well as those previously measured from our same control sites (−0.03 to 0.24 kg N_2O-N ha^{-1} yr^{-1} in 2008 and −0.34 to 0.48 kg N_2O-N ha^{-1} yr^{-1} in 2009; Martinson et al., 2013).

According to the hole-in-the-pipe (HIP) model, the amount of gaseous N loss from soils is primarily controlled by soil N availability, which is commonly measured using the soil N cycling rate (Firestone and Davidson, 1989; Davidson et al., 2000). We compared the net soil N cycling rates from our control plots (**Table 2**) with published data from other old-growth TMFs that used *in-situ* incubation of intact soil cores. Along our elevation gradient, net nitrification in the top 5 cm of soil was highest at 1000 m, likely reflecting the lower organic C and WFPS at 1000 m as compared to the higher elevations (Martinson et al., 2013). However, our net N cycling rates from 1000 m were lower than values reported for TMFs on Andosol soils located between 700 and 1500 m in northwestern Ecuador and Costa Rica (Arnold, 2008; Arnold et al., 2009). Similarly, net nitrification rates in the top 5 cm of the organic layer in an Andosol soil at 1200 m in Panama (Koehler et al., 2009) were more than 10 times higher than values from the same depth of organic layers in our Cambisol soil at 2000 m and our Histosol soil at 3000 m. A separate study carried out at our study sites showed that gross rates of mineral N production (N mineralization and nitrification) in our control plots were low and closely coupled with microbial N immobilization (Baldos et al., 2015), which is typical for conservative soil N cycling and supports our low net soil N cycling rates. Therefore, it is not surprising that the N_2O losses from our study sites were very low, with mean daily N_2O fluxes (**Table 1**) accounting for only 0.02–0.06% of gross N mineralization rates (used as an index of soil available N, ranging from 60 ± 10 mg N m^{-2} d^{-1} at 3000 m over 191 ± 53 mg N m^{-2} d^{-1} at 2000 m to 235 ± 30 mg N m^{-2} d^{-1} at 1000 m in the top 5 cm of soil; Baldos et al., 2015). This was comparable with the 0.06% N_2O loss in proportion to gross N mineralization rate in the top 5 cm of soil reported for a TMF in Panama (Corre et al., 2014).

The second level of control on gaseous N losses from soils in the HIP model is the soil aeration status, usually represented by the soil WFPS, which influences the relative contributions of nitrification and denitrification to gaseous N losses. Denitrification is proposed to become the dominant source of N_2O fluxes above a threshold value of 60% WFPS (Davidson et al., 2000) and to become the only N_2O source at WFPS >70% (Davidson, 1991; Machefert and Dise, 2004; Bateman and Baggs, 2005). Although, WFPS in the top 5 cm of soil only surpassed these threshold values at 2000 m (**Table 1**), the ^{15}N tracing method showed that denitrification was the dominant source of N_2O at 1000 m and the only N_2O source at 2000 and 3000 m. However, in previous studies, it has been shown that WFPS threshold values can vary substantially depending on soil texture; for example, in acid brown earth (Cambisol) with 48% sand in Northern Ireland, 60–80% of N_2O was derived from denitrification at 40% WFPS (Stevens et al., 1997). This is comparable to our results from the sandy loam mineral soil (Cambisol) at 1000 m. At 2000 and 3000 m, the 59–71% WFPS in the top 5 cm of the organic layer should theoretically have included some nitrification-derived N_2O fluxes. However, these organic layers had very high gravimetric soil moisture contents

(on average, 3.4 and 4.9 g g^{-1}) due to the high water holding capacity of the organic matter (Hudson, 1994). To illustrate this: approximately 27–30 kg H_2O m^{-2} was stored in the top 5 cm organic layer, which was much more than the approximately 15 kg H_2O m^{-2} stored in the top 5 cm of mineral soil at 1000 m. Such high gravimetric water contents in organic layers can create plenty of anaerobic microsites in which denitrification can occur despite the relatively low total WFPS. Indeed, the positive correlations between N_2O flux and NO_3^- (**Table 3**) also supported our results from the ^{15}N tracing method that denitrification was the dominant N_2O source. Thus, our findings illustrate that in contrast to mineral soils, different threshold values of WFPS should be used for organic layers in estimating limits of the relative importance of nitrification and denitrification as N_2O sources.

N-addition Effects on Soil N_2O Fluxes

In partial confirmation of our hypothesis, NO_3^- concentrations (at all elevations), as well as net ammonification (at 2000 and 3000 m), net nitrification (at 2000 and 3000 m) and NH_4^+ concentrations (at 2000 m), were all higher in N-amended (i.e., N and N+P) plots as compared to control plots (**Table 2**). These observed increases were supported by the increased gross rates of N mineralization and nitrification and decreases in microbial immobilization of NH_4^+ and NO_3^-, which were measured in the third and fourth year of nutrient manipulation at our sites (Baldos et al., 2015). However, in contrast to our hypothesis, these increases in net and gross rates of mineral N production and mineral N levels did not lead to increases in N_2O fluxes in N-amended plots relative to the control (**Table 1**; **Figure 1**). This does not mean that denitrification was not occurring, though, but may instead be an indication that produced N_2O was being further reduced to N_2. In the ^{15}N tracing experiment, although denitrification was the main N_2O source, emitted $^{15}N_2O$ accounted for maximally 0.065% of soil $^{15}NO_3^-$ (see *Results: effects of nutrient addition on N_2O*). This low percentage suggests that there may have been further reduction of N_2O to N_2. Reduction to N_2 is possible in soils, given favorable anaerobic microsites, high soil carbon and low soil NO_3^- levels (Weier et al., 1993). Such conditions were present in our sites, especially at the higher elevations with thick organic layers (see the discussion of water in organic layers above). Therefore, the combination of anaerobic microsites, with low NO_3^- concentrations (**Table 2**) and presumably high labile carbon in organic layers, may have resulted in high N_2/N_2O ratios, where losses via denitrification were dominated by N_2 (Weier et al., 1993). Chronic N addition can cause increases in NO_3^- levels and decreases in soil pH, which then inhibit N_2O to N_2 reduction; this was observed in an Andosol soil from a montane forest in Panama (Koehler et al., 2009, 2012; Corre et al., 2014). The increases in NO_3^- levels in our N-amended plots (**Table 2**), however, were much lower than those observed from the Panamanian montane forest soil, which received 4 years of 125 kg N ha^{-1} yr^{-1} (with resulting NO_3^- levels as high as 50–60 mg N m^{-2} in the organic layer and 112–183 mg N m^{-2} in the mineral soil). Our moderate levels of nutrient addition were probably the reason why soil pH in our N-amended plots did not yet differ significantly from the control plots even after 4 years (Baldos et al., 2015).

After the first 21 months of N addition to our sites, Martinson et al. (2013) reported that net nitrification at all elevations increased slightly and that these increases were accompanied by small increases in N_2O fluxes during the second year of N addition. Although we again observed higher N cycling with N addition in Months 35–56 (**Table 2**), we did not measure higher N_2O emissions with N addition (**Table 1**; **Figure 1**). Whether an increase in soil N availability (e.g., mineral N concentrations, net/gross rates of mineral N production) results in an increase in N_2O fluxes can depend on inter-annual variations in climate. In our results, the positive correlation of N_2O fluxes with WFPS at 1000 m, both in control plots (see *Results: control plots*) and across all treatments (**Table 4**), clearly indicates that there was a soil moisture control on N_2O fluxes. Additionally, at 2000 m, the correlations across treatments showed that a combination of changes in soil mineral N concentrations and moisture contents was controlling N_2O fluxes (**Table 3**). Corre et al. (2014) showed that the N_2O response to chronic N addition in tropical forest soils will tend to be more pronounced in wet years as opposed to dry years. They also showed that even small changes in moisture can strongly affect N_2O; a decrease of 7% in the seasonal average WFPS, corresponded to a 50% decrease in N_2O emissions (Corre et al., 2014).

Martinson et al. (2013) reported N_2O flux measurements from the initial 21 months of our study, but they had no information regarding the processes responsible for the observed emissions. Given the additional information from this study, we can now infer that the fluxes measured by Martinson et al. (2013) were predominantly denitrification-related and that gaseous N loss was likely dominated by N_2 rather than the small emissions of N_2O that they observed. We also know that WFPS from Months 35 to 56 (43–71%; **Table 1**) was lower than that measured from Months 1 to 21 of nutrient manipulation (63–88% WFPS; Martinson et al., 2013). Thus, the difference in the response of N_2O fluxes to N addition between the two study periods could be reflecting a general dampening of denitrification activity from the slightly wetter Months 1–21 to the slightly drier Months 35–56, which effectively removed the measureable response to N addition (i.e., the already low N_2O fluxes), while N_2 emissions may have continued to be elevated.

P-addition Effects on Soil N_2O Fluxes

As we hypothesized, we began to see P-addition effects on N_2O fluxes during our study period (Months 35–56), which were not present during the initial 2 years (Months 1–21) of nutrient manipulation (Martinson et al., 2013). At 1000 and 2000 m, soil N_2O fluxes in P plots were significantly lower than fluxes from N-amended plots, with the same trend—although not significant—when compared to control (**Table 1**; **Figures 1A,B**). This may have been related to changes in aboveground net primary production (ANPP) with P addition. Across our elevation gradient, ANPP was limited by P and/or co-limited by N+P, as shown by the trend toward higher basal area increment, which was already evident after 1 year of P addition (at 1000 and 2000 m) and N+P addition (at all elevations) (Homeier et al.,

2012, 2013). If P addition increased ANPP, there may have been an increase in plant uptake of other soil nutrients, including soil mineral N. Since P addition did not change net (**Table 3**) or gross (Baldos et al., 2015) rates of mineral N production, an increase in uptake of soil mineral N by plants would lead to lower mineral N levels in P plots; although not always significant, we did measure lower mineral N concentrations (especially NO_3^-) in soils of P plots at 1000 and 2000 m (**Table 2**). Since NO_3^- was the main substrate for N_2O production across our elevation gradient, decreased NO_3^- concentrations in P plots may have led to reduced N_2O fluxes. Similar results were observed in a 6-year old leguminous tree plantation in Indonesia, where P addition alleviated plant P limitation and increased root N uptake, resulting in decreased mineral N concentrations and N_2O fluxes (Mori et al., 2013).

At 3000 m, we possibly observed the same mechanism (i.e., P addition catalyzing N uptake) with slightly different results. At this elevation, there were significant decreases in NH_4^+ and NO_3^- concentrations (**Table 2**) with no significant change to N_2O fluxes. We attribute this to the fact that initial fluxes of N_2O were too low to detect a decrease (**Table 1**; **Figure 1C**). This idea is supported by the fact that N_2O fluxes at 3000 m were not correlated with any of the measured soil factors, neither for control plots nor across all treatments (**Tables 3, 4**), which again suggests that the N_2O fluxes were too low (mostly fluctuating around zero; **Figure 1C**) to generate any significant relationships with the soil factors known to control N_2O fluxes.

As shown in the N-addition section above, the effects of N+P addition on net N cycling and soil mineral N concentrations followed the same trends as those for N addition alone (**Table 2**). However, it is notable that the increases as a result of N addition in the N+P plots were not as strong as the increases in the N plots, presumably because of the opposing effect of P addition. Although N-cycling responses to P addition appeared to be delayed as compared to responses to N addition (i.e., there were no significant effects of P addition during the initial 21 months reported by Martinson et al., 2013), the presence of P in atmospheric deposition could be an important long-term control on N_2O fluxes in ecosystems where deposition of both nutrients is occurring. For example, although our study did not show any nutrient-addition effects on N_2O fluxes, Baral et al. (2014) in a different study related to N_2O fluxes also observed that added P addition increased plant N uptake; in their case this resulted in significantly less N_2O emissions with N+P addition as compared to N addition alone.

CONCLUSION

We have shown that soil N_2O fluxes in our study sites were among the lowest measured in TMFs and that denitrification was the main source of N_2O, which was possibly being produced in anaerobic microsites. We attribute the low N_2O fluxes to the conservative soil N cycling along our elevation gradient (Baldos et al., 2015), and the combination of low NO_3^- concentrations and presumably high available C in the organic layers (at 2000 and 3000 m) which could favor the already low gaseous N losses to be dominated by N_2. In contrast to the first 21 months of this study (Martinson et al., 2013) we did not detect significant increases in N_2O fluxes in Months 35–56, despite an increase in soil N availability. This may be due to the generally low N_2O fluxes during our measurement period, which we in turn attribute to the lower rainfall and soil moisture contents during our study period. However, we did detect a reduction in soil mineral N concentrations and N_2O fluxes with P addition, in contrast to the first 21 months when no effects were observed (Martinson et al., 2013). The significant P effect during our study period was probably due to increased uptake of soil mineral N by vegetation after an extended period of P addition, since P is a limiting element for ANPP at our sites (Homeier et al., 2012, 2013). Nitrogen plus phosphorus addition showed similar trends in net rates of mineral N production, mineral N concentrations and N_2O fluxes to those with N addition alone, although to a lesser degree because of the counteracting effects of P addition. This 5-year study (the work of Martinson et al., 2013 together with our results), strongly illustrated that effects of nutrient addition on soil N_2O fluxes are not always linear with time of exposure. We observed large inter-annual variation in N_2O responses, which we primarily attributed to changes of soil moisture conditions, combined with soil characteristics such as texture and organic C content. Without this multiple-year study we would not have been able to detect these changes in nutrient response over time.

AUTHOR CONTRIBUTIONS

Substantial contributions were achieved by all listed authors and were as follows:

Conception and design of the work: AKM, MC, EV
Acquisition of data: AKM, ALM
Analysis and Interpretation of data: AKM, ALM, MC, EV
Drafting and revising the work: AKM, ALM, MC, EV.

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SUPPLEMENTARY MATERIAL

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Performance of Seedlings of a Shade-Tolerant Tropical Tree Species after Moderate Addition of N and P

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Nitrogen deposition to tropical forests is predicted to increase in future in many regions due to agricultural intensification. We conducted a seedling transplantation experiment in a tropical premontane forest in Ecuador with a locally abundant late-successional tree species (*Pouteria torta*, Sapotaceae) aimed at detecting species-specific responses to moderate N and P addition and to understand how increasing nutrient availability will affect regeneration. From locally collected seeds, 320 seedlings were produced and transplanted to the plots of the Ecuadorian Nutrient Manipulation Experiment (NUMEX) with three treatments (moderate N addition: 50 kg N ha^{-1} $year^{-1}$, moderate P addition: 10 kg P ha^{-1} $year^{-1}$ and combined N and P addition) and a control (80 plants per treatment). After 12 months, mortality, relative growth rate, leaf nutrient content and leaf herbivory rate were measured. N and NP addition significantly increased the mortality rate (70 vs. 54% in the control). However, N and P addition also increased the diameter growth rate of the surviving seedlings. N and P addition did not alter foliar nutrient concentrations and leaf N:P ratio, but N addition decreased the leaf C:N ratio and increased SLA. P addition (but not N addition) resulted in higher leaf area loss to herbivore consumption and also shifted carbon allocation to root growth. This fertilization experiment with a common rainforest tree species conducted in old-growth forest shows that already moderate doses of added N and P are affecting seedling performance which most likely will have consequences for the competitive strength in the understory and the recruitment success of *P. torta*. Simultaneous increases in growth, herbivory and mortality rates make it difficult to assess the species' overall performance and predict how a future increase in nutrient deposition will alter the abundance of this species in the Andean tropical montane forests.

Keywords: Ecuador, tree seedlings, diameter growth, herbivory, foliar nutrients, nutrient manipulation experiment, *Pouteria torta*, seedling transplantation experiment

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INTRODUCTION

Phosphorus (P) and nitrogen (N) are supposed to limit stand productivity in the majority of terrestrial ecosystems as well as in tropical forests (Tanner et al., 1998; Elser et al., 2007; Vitousek et al., 2010; Harpole et al., 2011; Homeier et al., 2012). Anthropogenic influences have modified natural nutrient availability worldwide by pollutants released to the atmosphere and the resulting increased deposition to soils. It is expected that increasing nutrient deposition has the potential to alter the dynamics of tropical forest ecosystems causing structural and compositional changes

of the plant communities in response to modified availability of limiting elements (e.g., Lewis and Tanner, 2000; Homeier et al., 2012).

Nutrient effects on tropical forests largely depend on the local conditions; factors such as temperature, precipitation, soil type and pedogenesis, and local biota determine overall nutrient availability at a specific site and if an element is limiting plant growth. Thus, for example tropical lowland rainforests growing on old, highly weathered soils under humid conditions at high temperatures are most likely to be P limited, whereas tropical montane forests that are typically exposed to a cooler and wetter climate most often grow on younger or rejuvenated soils where N is likely to limit plant growth (Tanner et al., 1998; Unger et al., 2010; Vitousek et al., 2010; Wolf et al., 2011; Fisher et al., 2013). In general, terrestrial ecosystems in many cases seem to be limited by both N and P. Hence, addition of either N or P or of both elements is stimulating plant growth (Elser et al., 2007; Harpole et al., 2011).

Since compositional shifts in communities of long-lived organisms like trees need decades to become visible, studies on mature trees will rarely provide information about future changes in forest dynamics caused by increased nutrient availability. In fact, seedlings of tropical rainforest trees have been found to respond more sensitively to changes in nutrient availability (e.g., Lawrence, 2003; Yavitt and Wright, 2008; Dent and Burslem, 2009; Andersen et al., 2010; Lu et al., 2010; Holste et al., 2011) due to the strong physiological dependence of this stage on resource availability in a highly competitive environment (Kitajima, 1996).

It is known that processes affecting the seedling stage act as a strong selective filter controlling patterns of recruitment and are thereby influencing the future species composition of the forest (Swaine, 1996; Whitmore, 1996; Metz et al., 2008). Therefore, it is crucial to understand the impact of changed nutrient availability at this stage of the life cycle. Several studies have used seedlings of locally abundant tropical tree species to study if these species are able to optimize their resource use after nutrient addition (e.g., Lawrence, 2001; Yavitt and Wright, 2008; Andersen et al., 2010; Pasquini and Santiago, 2012; Alvarez-Clare et al., 2013) and concluded that changes in establishment and growth rates might be considered as a key driver of structural changes of tree communities to altered nutrient availability.

Responses to changed nutrient availability likely will depend on the species' life strategy. Some species are expected to rapidly invest extra nutrients to increase growth especially under favorable light conditions, whereas others rather may store nutrients in their tissues (e.g., Huante et al., 1995b; Raaimakers and Lambers, 1996; Lawrence, 2003). Biomass allocation varies, depending on how seedlings accumulate carbohydrates to aboveground tissues vs. root surface (Burslem et al., 1995; Wan Juliana et al., 2009). Carbon gain and plant growth are highly dependent on leaf traits, in particular photosynthetic capacity and leaf area, which both tend to increase with nutrient addition (Burslem et al., 1995; Wan Juliana et al., 2009; Pasquini and Santiago, 2012).

Large increases in nutrient availability, however, may also lead to a decrease in density and diversity of the seedling community by altering survival and establishment rates of a share of species because mortality could be altered by increased herbivory, e.g., as a consequence of N or P accumulation in leaf tissues (Andersen et al., 2010; Santiago et al., 2012). In addition, seedling establishment might be impeded due to soil acidification (Lu et al., 2010) or due to modified competition with other species (Lawrence, 2001).

To study the effects of increased nutrient availability on a locally abundant climax species, we took advantage of the ongoing nutrient manipulation experiment NUMEX in Ecuador. NUMEX was started in 2008 to study if and to what extent N and P limitation are controlling tropical montane forest functioning at three elevation levels (1000, 2000, and 3000 m) and to simulate the effects of future increasing nutrient availability from atmospheric deposition on different ecosystem processes. In southern Ecuador, nutrient deposition has been suggested to largely origin from extensive biomass burning occurring in the Amazon basin leading to increasing depositions of >10 kg N ha^{-1} in the last decade (Fabian et al., 2005; Wilcke et al., 2013). The continued nutrient addition in this experiment had already shown effects on various belowground to aboveground compartments in the experiment's first years: Homeier et al. (2012) found in the common tree species a positive growth response after N (two out of four species), P (two out of four species) and N+P addition (three out of four species) and an increase of leaf litter production after N and NP addition suggesting that at 2000 m. a.s.l. N and P might be co-limiting. At the same site, Camenzind et al. (2014) detected changes in the abundance and species richness of arbuscular mycorrhizal fungi (AMF) after N and P addition. At 1000 m. a.s.l., four years of N and N+P addition (but not P addition alone) resulted in increased mineral N production and decreased microbial N retention and microbial biomass C and C:N ratio (Baldos et al., 2015). N_2O emissions were only increased during the first two years of N and NP addition (but not P addition alone), and P addition tended to reduce soil N cycling and to decrease N_2O fluxes (Martinson et al., 2013; Müller et al., 2015). Morevover, P (but not N) additions increased the amount of water stable soil macroaggregates, which play an important role for soil stability and nutrient cycling (Camenzind et al., submitted). Krashevska et al. (2014) found that the decomposer system (microorganisms and saprophytic fungi) of the studied NUMEX plots responded to the changes in nutrient inputs concluding that at all elevations microorganisms were generally limited by N and saprophytic fungi also by P. In addition, P and N+P treatments increased the bioavailable inorganic P concentrations and decreased phosphatase activity (Dietrich et al., submitted).

At the premontane study site at 1000 m. a.s.l. we found an average foliar N:P ratio of 28.0 in 80 unfertilized trees from 25 different plant families sampled at mid slope position (Homeier et al., unpublished data). We assume that P is the prevailing limiting nutrient at this site, since according to Townsend et al. (2007) N:P ratios >16 suggest P-limitation. The two other NUMEX sites show also average foliar N:P ratios >16 (2000 m. a.s.l.: N:P ratio $= 22.1$, 78 trees from 31 plant families; 3000 m. a.s.l.: 26.0, 65, 16; Homeier et al., unpublished data). Thus, the foliar N:P ratio does not indicate a shift to N limitation toward higher elevations.

To test the response of seedlings of the locally most abundant climax tree species *Pouteria torta* to continued nutrient addition, we transplanted 9-month-old seedlings to the NUMEX experimental plots at 1000 m. a.s.l. in 2011. Our investigation aimed to test how continued nutrient addition (a) will alter mortality and growth rates in seedlings, (b) whether leaf morphology, folivory, and foliar nutrient concentrations will indicate changed nutrient use (e.g., higher area loss and high foliar N concentration after N addition) and (c) how biomass partitioning to above and belowground organs will change as a response to extra nutrient availability.

We aimed to understand how N and P limitation are influencing seedling growth and vitality in this abundant species and how its dominant role in the forest might change under increasing nutrient deposition. Based on earlier findings on the nutrient response of shade-tolerant tropical tree seedlings, we hypothesized that *Pouteria torta* seedlings after being released from potential nutrient limitation would respond primarily by accumulating N and P in the biomass rather than by increasing its growth rate.

MATERIAL AND METHODS

Study Site

The study was conducted in Podocarpus National Park, southern Ecuador (province Zamora-Chinchipe), close to the Bombuscaro entrance (S 4° 7′, W 78° 58′) in the transition zone between tropical lowlands and lower montane forests. Vegetation has been described as evergreen premontane forest with tree heights up to 40 m (Homeier et al., 2008), but average stand height is between 20 and 25 m. Most abundant tree families are Fabaceae, Melastomataceae, Moraceae, Myristicaceae, Rubiaceae, and Sapotaceae; common tree species are *Pouteria torta* (Mart.) Radlk. (Sapotaceae) and *Clarisia racemosa* Ruiz & Pav. (Moraceae; Homeier et al., 2008, 2013).

Mean annual temperature at the study site is around 20°C and annual mean precipitation ∼2200 mm, without a clear seasonal pattern (Emck, 2007; Peters, unpublished data). Soils are Dystric Cambisols developed from deeply-weathered granodioritic rock of the Jurassic Zamora granitoide formation (Wolf et al., 2011). The mineral soil is covered by a thin layer of decomposing leaves (Ol layer) (Homeier et al., 2013). Soil characteristics (means ± SE) of the mineral topsoil (0–5 cm) after 4 years of nutrient addition (April 2012) are summarized in Supplementary Table 1.

Study Species

The locally most abundant tree species of the old-growth forests at Bombuscaro, *Pouteria torta* subsp. *glabra* T. P. Penn., is a late successional shade-tolerant tree species (Wittich et al., 2015; Homeier, own observations), accounting for 9.5% of the stems ≥ 10 cm dbh or about 69 stems ha^{-1} in the NUMEX plots. It reaches heights up to 30 m and about 50 cm stem diameter and its wood specific gravity is high with 0.79–0.87 g cm^{-3} (Homeier, unpublished data). The species is widely distributed in the Neotropics from Colombia to the Guianas and in western South America to Bolivia. It is known to occur from the lowlands up to 1500 m. a.s.l. (Pennington, 2007).

Experimental Design

The full-factorial Ecuadorian nutrient manipulation experiment (NUMEX) was set up at 990–1100 m.a.s.l. in a stratified random design which consists of four blocks with four treatments (control, N, P, and N+P addition) assigned to every block (16 experimental plots). Experimental plots (20×20 m) were installed in old-growth forest with closed canopy at mid slope position; the plots are separated from each other by at least 10 m-wide strips of untreated forest (Supplementary Figure 1).

Fertilizations started in February 2008. Fertilizers are applied by hand to the experimental plots at rates of 50 kg N ha^{-1} (as urea) in the N treatment plots, or 10 kg P ha^{-1} (NaH_2PO_4) in the P treatment plots, or 50 kg N and 10 kg P (NP treatment plots) applied in two portions per year (Homeier et al., 2013). For the seedling experiment, four new subplots of 1 m^2 were randomly placed into each NUMEX experimental plot (64 subplots in total). Irradiance at the forest floor was quantified in every subplot with hemispherical photos (Nikon D5000 camera with 8 mm-fish eye lens) taken at the midpoint 1 m above ground under uniform sky conditions in 2012. Images were analyzed using Gap Light Analyzer 2.0 software (Frazer et al., 1999). Light availability in the subplots indicated a mean canopy openness of 8.85 ± 4.1(SD) % with a range of 1.25–19.48%.

Seedlings of *Pouteria torta* were raised after a massive fruiting event that occurred in 2010. More than 400 seeds from about 15 trees were collected in September 2010 at the study site and sown in a greenhouse supplied by a local farmer. In June 2011, 320 seedlings were selected and transported to the study site. All seedlings were measured for total height and labeled before planting; mean initial height was 17.4 ± 4.9 (SE) cm (**Table 1**). At that time, there were no cotyledons left on the plants and seedlings had completely consumed the reserves stored in the seeds.

Five seedlings were assigned to each experimental subplot. Because of small differences in initial height, we classified seedlings into height categories before planting and then equally distributed them to the 64 subplots to ensure a similar seedling height distribution in each plot. Subplots were also cleaned from other tree seedlings and ground herbs to avoid root competition.

TABLE 1 | Properties of the initial seedling cohort before transplantation to the NUMEX plots in June 2011.

Seedling properties	Treatments			
	Control	N	P	N + P
Height (cm)	17.2 (9.2–31.0)	16.8 (5.5–27.0)	18.1 (9.5–30.0)	17.4 (6.5–29.0)
Diameter (mm)	3.0 (2.0–4.1)	3.0 (1.8–4.0)	3.0 (1.7–5.2)	3.1 (1.9–4.5)
Leaf number	8.0 (3–19)	7.3 (1–18)	8.0 (1–18)	7.4 (2–17)
Leaf length (cm)	6.7 (1.1–14.8)	7.4 (2.2 – 17.0)	6.7 (1.4–15.0)	7.6 (1.4–17.0)
Leaf width (cm)	2.2 (0.5–4.0)	2.3 (0.4–5.5)	2.1 (0.4–4.5)	2.5 (0.5–5.0)

Given are means and ranges (in parentheses).

Once seedlings were planted, initial size characteristics were measured (stem height and basal diameter, leaf number, length, and width of the largest leaf).

After 1 year, in May 2012, all surviving seedlings were carefully harvested after a final assessment of basal diameter and leaf area loss. We excavated all seedlings, removed all soil from the roots, and then separated the plants into root, stem and leaf fractions. Fresh leaves were scanned immediately after harvesting seedlings (color, 150 dpi) with a Cannon LIDE 1000 flatbed scanner. All seedling fractions were dried for 72 h at 60°C and then transferred to Germany.

Leaf Analyses

The scanned images of the *Pouteria* leaves were analyzed using Win Folia 2005b software (Régent Intruments Inc., Quebec City, QC, Canada) to calculate total leaf area including damaged areas (hole area). Subsequently, images were manually adjusted by filling in missing leaf sections, to estimate the original leaf area prior to damage. Specific leaf area (SLA, $cm^2\ g^{-1}$) was calculated for each seedling by dividing the entire remaining leaf area of all leaves by its mass. Proportional leaf area loss was determined as an estimate of herbivory rate, the damage inflicted over the life span of the leaves with the following formula:

$$\text{Leaf area loss (\%)} = \text{damaged area (cm}^2\text{)/total original leaf area(cm}^2\text{)}$$

Leaf length ratio and leaf width ratio were calculated by dividing the respective dimension of the biggest leaf at harvest and before planting to estimate changes in leaf dimensions during our experiment:

$$\text{Leaf length ratio} = \text{final lamina length (cm)/initial lamina length (cm)}$$

The leaf area ratio (LAR) was calculated as the amount of leaf area per unit of total plant mass.

Nutrient Analyses

We determined total N and C concentrations of the leaf mass with a C/N elemental analyzer (Vario EL III, Elementa, Hanau, Germany). Total P concentrations were analyzed using an Inductively Coupled Plasma Analyzer (Optima 5300DV ICP-OES, Perkin Elmer, Waltham, Massachusetts, USA) after digesting the leaf samples with concentrated HNO_3. Nutrient analyses were conducted at the laboratory of the Department of Ecology and Ecosystems Research, University of Göttingen, Germany for all individual seedlings harvested at the end of the experiment.

Data Treatment and Statistical Analyses

Mortality rates were calculated from the proportion of dead individuals in the subplots (i.e., number of dead seedlings divided by seedlings planted). Effects of nutrients on mortality were tested with a generalized linear mixed model (GLMM) for a binomial distribution using counts of the dead individuals in a full factorial approach using the function "glmer" in the lme4 package (Bates et al., 2014) with N and P as fixed effects and "block" as a random factor. Significance values were obtained after testing the model with the "cftest" function from the multcomp package (Hothorn et al., 2008). This function calculates, in a univariate testing way, the *p*-values from estimated model coefficients obtained from a *z*-test. The relative stem diameter growth rate (RGR_D) was calculated from diameter measurements for the study interval as:

$$(\ln(d_2) - \ln(d_1))/(t_1 - t_0),$$

where d_2 represents the final diameter and d_1 the initial diameter, and t_1-t_0 represents the time span between planting and harvesting dates.

The mass fractions for leaves (LMF), stems (SMF) and roots (RMF) were calculated by relating organ mass to total plant mass for each seedling following Poorter et al. (2012).

Data were transformed to reduce heteroscedasticity when required (log transformations for biomass ratios, leaf area and leaf nutrient contents and square root transformations for biomass, leaf fractions, and leaf area loss).

To test specifically how nutrients are influencing seedling performance, we used a full factorial linear mixed model (Zuur et al., 2009) with N and P as fixed effects using the "lme" function of the nlme package (Pinheiro et al., 2014) for all seedlings measured and harvested in 2012 ($n = 124$). For all parameters assessed (RGR_D, SLA, leaf area loss, foliar N/P, and C/N ratio, root/shoot and root/leaf ratios, LAR, biomass fractions, foliar nutrient contents), canopy openness was evaluated first in order to check for interaction with fixed effects (treatment). Since it had no influence on the responses to nutrient addition, canopy openness was not included in the final models. In all models, we included "block" as a random factor.

All comparisons contrasted differences between treatments (N, P or interaction of N and P) with control. All analyses and figures were done with R 3.1.1 software (R Development Core Team, 2014). Specific information about the structure of the fitted models all parameters analyzed is summarized in Supplementary Table 2.

RESULTS

Seedling Mortality and Growth

The seedlings suffered high mortality (**Figure 1**); only 124 ($con = 37$, $N = 24$, $P = 39$, $NP = 24$) of the initially planted 320 *Pouteria torta* seedlings survived the first year. On average, 54% ($n = 43$) of all seedlings in the control and 51% ($n = 41$) of those in the P treatment died. N fertilization increased seedling mortality significantly ($p = 0.016$) to 70% ($n = 56$) in both N treatments compared with the control (**Table 2**).

The relative diameter growth rate (RGR_D) of the surviving seedlings was 0.23 $year^{-1}$ in the control subplots (**Figure 2**). N fertilization ($p = 0.006$) and P fertilization ($p = 0.021$) significantly increased diameter growth by 30–50% compared to the control.

Leaf Properties and Herbivory

Leaf area showed no significant difference between treatments although largest leaves were found in the N and NP treatments (**Table 2**). Compared to the control, SLA showed a significant increase of more than 40% after N addition (**Figure 3**, **Table 2**). In addition, we observed higher leaf length and leaf width ratios after N addition, whereas leaves in the control and in the P addition plots did not increase their dimensions.

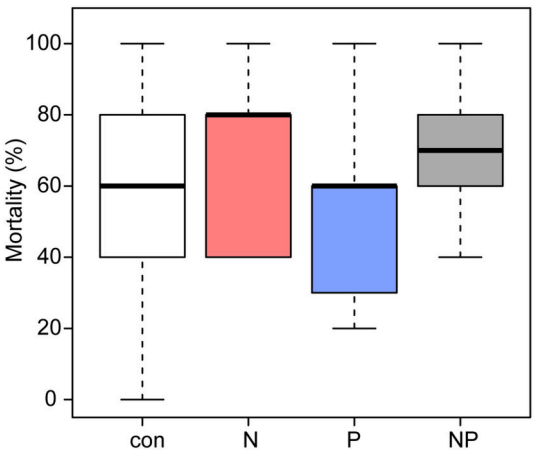

FIGURE 1 | Mean percentages of mortality in *Pouteria torta* seedlings observed during 1 year of exposure to moderate nutrient additions (2011–2012). Addition of N and of N + P resulted in a significantly higher mortality of the seedlings ($p = 0.016$).

Seedlings grown in the P and NP treatments had highest leaf area losses compared with seedling in control plots indicating a stimulation of herbivory by P fertilization (**Figure 3**, **Table 2**).

Mass based foliar N and P concentrations were only weakly affected by the fertilization since we found only marginally higher foliar N after N addition, but the foliar C/N ratio was lowered (**Figure 3**). Area-based foliar N and P concentrations were lower in the N treatment.

N and P foliar contents on a mass basis (i.e., total foliar nutrient content per plant) showed no differences between treatments. The mean values of foliar N/P ratios varied between the treatments from 15.9 in the control to 17.9 in the NP treatment.

Biomass Allocation

Across all treatments, *Pouteria* seedlings allocated most of their biomass to the roots (**Table 2**, **Figure 4**). P addition shifted allocation even more to roots as expressed by an increasing root mass fraction (P: 55%, NP: 56% vs. control: 51%) and a higher root/shoot ratio (P: 1.87, NP: 2.07 vs. control: 1.69).

TABLE 2 | Summary of the properties of surviving *Pouteria torta* seedlings after 1 year.

Parameter	Treatment				Effect		
	Control mean (SE)	N mean (SE)	P mean (SE)	N + P mean (SE)	N	P	N × P
Mortality (%)	0.54 (0.05)	0.70(0.03)	0.51 (0.04)	0.70 (0.03)	+*		
Range of Mortality (%)	35–75	60–80	40–65	60–85			
RGR_D (mm mm^{-1} $year^{-1}$)	0.23 (0.02)	0.39 (0.02)	0.34 (0.03)	0.37 (0.03)	+**	+*	−*
LA (cm^2)	13.44 (1.05)	15.21 (2.45)	14.85 (1.06)	16.70 (2.01)			
Leaf length ratio	0.96 (0.11)	1.05 (0.12)	0.85 (0.08)	1.00 (0.10)			
Leaf width ratio	0.99 (0.13)	1.15 (0.14)	0.88 (0.08)	1.09 (0.10)	+*		
SLA (cm^2 g^{-1})	68.2 (1.0)	103.7 (1.7)	73.5 (1.0)	100.3 (1.6)	+**		
Leaf area loss (%)	5.16 (0.87)	4.87 (1.03)	8.88 (1.85)	10.14 (2.22)		+*	
Foliar N (mg g^{-1})	22.04 (0.63)	22.77 (0.63)	21.33 (0.50)	22.90 (0.82)			
Foliar P (mg g^{-1})	1.44 (0.08)	1.39 (0.11)	1.41 (0.08)	1.39 (0.10)			
Foliar N (mg cm^{-2})	0.46 (0.04)	0.32 (0.03)	0.44 (0.04)	0.35 (0.05)	−**		
Foliar P (mg cm^{-2})	0.03 (0.002)	0.02 (0.003)	0.03 (0.003)	0.02 (0.01)	−**		
Foliar N (mg) per seedling	5.62 (0.60)	4.54 (0.75)	5.83(0.78)	5.48(1.04)			
Foliar P (mg) per seedling	0.38 (0.04)	0.32 (0.06)	0.40 (0.05)	0.38 (0.09)			
Foliar N:P ratio	15.90 (0.72)	17.15 (0.76)	16.51 (0.82)	17.90 (1.31)			
Foliar C:N ratio	22.62 (0.61)	21.69 (0.66)	23.55 (0.53)	21.73 (0.73)	−*		
Total biomass (g)	1.63 (0.10)	1.65 (0.12)	1.77 (0.13)	1.86 (1.15)			
LMF	0.16 (0.02)	0.12 (0.01)	0.16 (0.02)	0.13 (0.02)	−*		
SMF	0.32 (0.01)	0.35 (0.01)	0.29 (0.02)	0.32 (0.02)	+*	−**	
RMF	0.51 (0.02)	0.53 (0.02)	0.55 (0.02)	0.56 (0.01)		+*	
Root: shoot ratio	1.69 (0.09)	1.58 (0.08)	2.07 (0.12)	1.87 (0.11)		+**	
Root: leaf ratio	7.82 (2.60)	10.04 (2.66)	6.98 (1.22)	7.87 (1.54)			
LAR	8.38(0.62)	8.95(1.17)	8.95(0.61)	9.62(0.77)			

The direction of significant effects (compared to the control treatment) is indicated (±) ($p \leq 0.05$; **$p \leq 0.01$).*

The leaf mass fraction of the seedlings in the N treatments was significantly reduced (N: 12%, NP: 13% vs. control: 16%) and the stem mass fraction was higher after N addition (N: 0.35 vs. control: 0.32). Mean LAR was slightly but not significantly higher in the fertilized plots compared to the control.

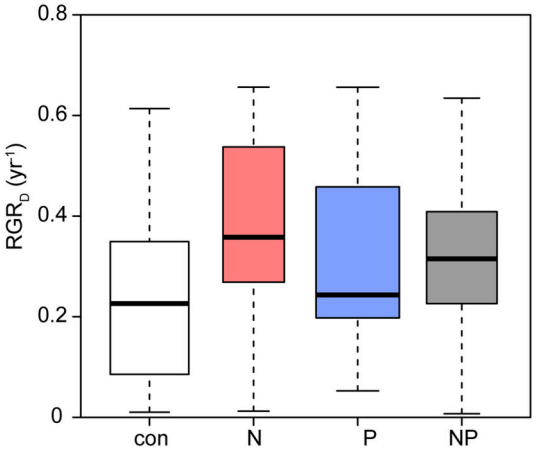

FIGURE 2 | Relative diameter growth rates of *Pouteria torta* seedlings as a response to moderate nutrient addition. Single addition of N or P caused increased growth rates (N: $p = 0.006$; P: $p = 0.021$), interaction of both nutrients resulted in a smaller effect than when the elements were added alone (N × P: $p = 0.017$).

DISCUSSION

Seedling Mortality and Growth

During the life cycle of a tree, the seedling stage is the most vulnerable phase with susceptibility to physical harm (e.g., litterfall, trampling, herbivory, or pathogen attack) and carbohydrate shortage (Alvarez-Clare and Kitajima, 2007, 2009). Seedling mortality is highly related to the plants' physical and physiological robustness to withstand adverse conditions in order to establish successfully (Kitajima and Fenner, 2000; Alvarez-Clare and Kitajima, 2007).

Under natural forest conditions, seedling transplantation experiments in tropical forests commonly experience high mortality during the first 2 months after seedling transplantation (Eichhorn et al., 2006; Alvarez-Clare and Kitajima, 2009; Brenes-Arguedas et al., 2011), which is mainly attributed to damage by herbivores and defoliation (Paine et al., 2012). In our study, *Pouteria torta* experienced a high mortality of >50% in the first year after planting, although transplantation occurred 6 months after germination when we assumed that the seedlings already had reached a "robust" stage.

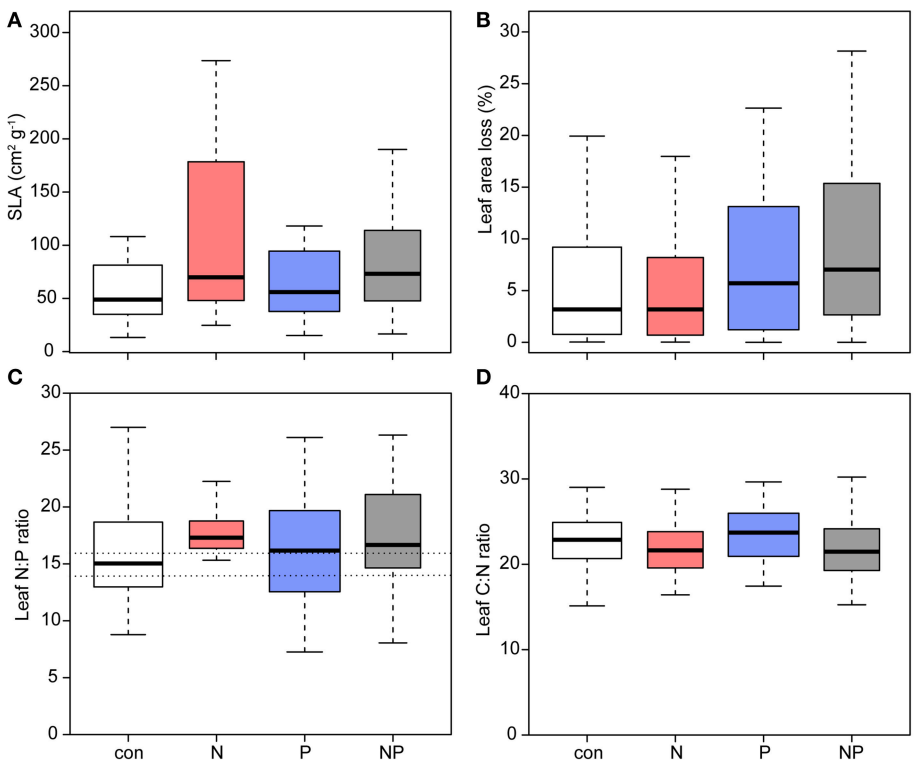

FIGURE 3 | Leaf morphology, leaf area loss and nutrient ratios of *Pouteria torta* seedlings after 1 year of experimental exposure to nutrient addition. **(A)** Specific leaf area (SLA) increased after N addition ($p = 0.009$), **(B)** the proportion of lost leaf area showed a significant increment after P addition ($p = 0.020$), **(C)** foliar N:P ratio was not affected in the treatments (dotted lines mark the range of N:P ratios indicating a balanced supply of both nutrients after Townsend et al., 2007), and **(D)** leaf C:N ratio was decreased in the two N treatments ($p = 0.024$).

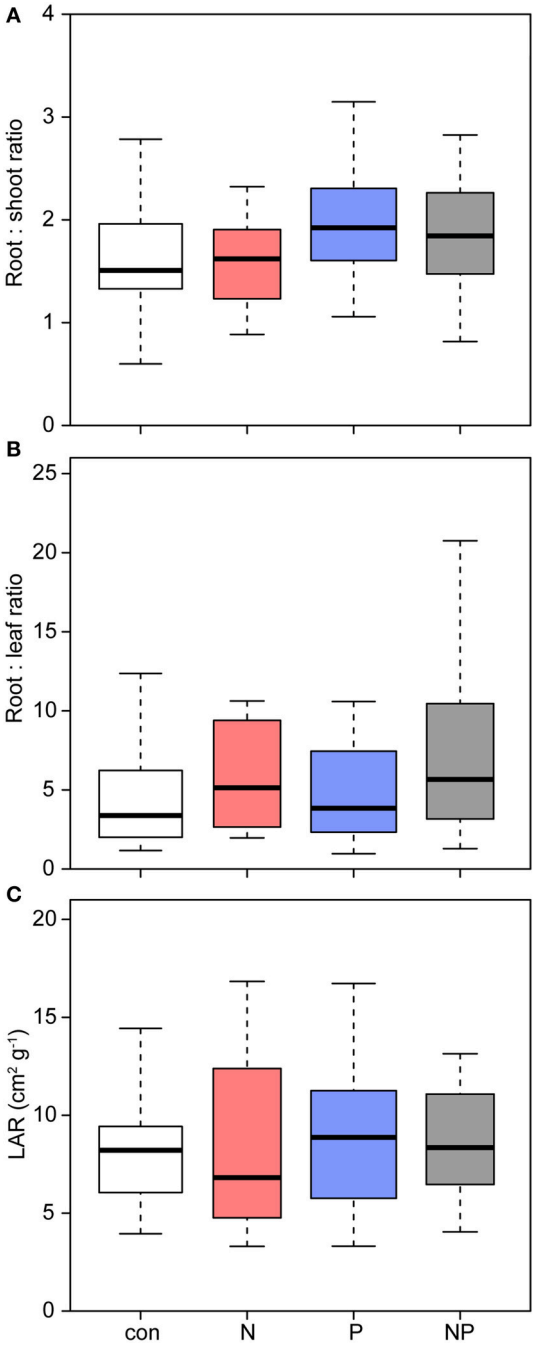

FIGURE 4 | Effects of nutrient addition on the biomass allocation of *Pouteria torta* seedlings. (A) Changes in the root:shoot ratio indicate a shift in allocation toward the roots after P addition ($p = 0.003$), **(B)** N addition increased the root:leaf ratio marginally ($p = 0.061$), **(C)** but the leaf area ratio (LAR) was not affected.

The increase in mortality in the two N treatments is contrary to results of Vincent and Tanner (2013) who reported no reduction of survival rate in transplanted seedlings of two late-successional tree species after NPK addition in a Panamanian lowland forest. Since there was no significant change in soil pH in our plots after 4 years of nutrient addition (Supplementary Table 1; Baldos et al., 2015), we cannot argue with impeded seedling establishment by soil acidification as reported by Lu et al. (2010).

In spite of the likely complex interaction among drivers of seedling growth under natural forest conditions, we found positive effects of either N or P addition on diameter growth of the surviving *Pouteria* seedlings. Both elements N and P are essential for photosynthesis, since ribulose 1-5-bisphosphate oxygenase/carboxylase (RuBisCO) protein accounts for ∼30% of leaf N, and P plays an important role in membrane solubility, ATP, and NADPH production (Marschner, 1995; Walker et al., 2014). Since foliar N and P did not increase while SLA increased, an increase in photosynthetic rate cannot be the cause of higher productivity; rather, the (non-significant) increase in plant leaf area must have led to the higher productivity in the fertilized plants. Several other pot experiments with tropical tree seedling showed positive effects of N and/or P addition on growth as well but other studies reported no effects (see compilation in Lawrence, 2003). In general, tree growth limitation by N and P strongly depends on specific site conditions and on co-limitation by other nutrients. Santiago et al. (2012) showed a 24% increase in relative height growth in naturally established tropical tree seedlings as a response to K addition and a smaller but also positive effect of combined long-term addition of N and P. Increased growth rates are probably the result of enhanced photosynthetic productivity as reported by Pasquini and Santiago (2012) for *Alseis blackiana* seedlings from the same tropical lowland forest in Panama.

Leaf Properties and Herbivory

Leaf properties are recognized as key traits in determining establishment and survival success of tree seedling under varying site conditions and they are usually a good indicator of potential growth rates. There is a strong trade-off between high leaf lifespan, high leaf toughness and low herbivory and low mortality on the one hand, and high foliar nutrient concentrations and fast growth rates on the other hand (e.g., Poorter and Bongers, 2006; Wright et al., 2010; Kitajima et al., 2013; Philipson et al., 2014).

We observed a strong positive effect of N addition on SLA. Other studies reported a higher SLA in tropical tree seedlings after P addition (e.g., Burslem et al., 1995: *Antidesma cuspidatum* in Singapore, Wan Juliana et al., 2009: *Lagerstroemia floribunda* in Malaysia) or found no effect of fertilization on SLA (Santiago et al., 2012: five shade-tolerant tree species). SLA is known to be a good predictor of growth rates in seedlings (Wright and Westoby, 1999, 2000), so the increased growth rates in the N treatments are not surprising. Strong effects of herbivory in the P treatments were probably masking an even larger productivity stimulation by the nutrient addition (especially in the NP treatment, where herbivory and also SLA were significantly increased compared to the control); a similar masking of growth increase by herbivory was shown by Andersen et al. (2010) for different palm species after N addition. Increased leaf damage through herbivory after P addition was also reported by Santiago et al. (2012) in Panama where the proportion of herbivory in seedlings was higher after P or K addition. Werner and Homeier (2015) found a

positive correlation of eaten leaf area with both foliar N and P concentrations in a montane forest close to our study site. All these studies show that herbivore choice is strongly driven by resource quality (i.e., nutrient content and palatability of leaves). Hence, herbivory can play a fundamental role in structuring seedling communities by directly affecting the survival of plants or mediating responses to soil nutrient availability (e.g., Andersen et al., 2010; Eichhorn et al., 2010; Barton and Hanley, 2013).

Leaf tissue N:P ratios of 15.9 in the control plots point at a balanced supply of both nutrients (Townsend et al., 2007) and after addition of N or P, *Pouteria* seedlings did not accumulate the respective nutrient in their leaves. This is contrary to the results of several studies that found an increase of foliar nutrient concentrations in shade-tolerant tree seedlings after fertilization (Burslem et al., 1995; Lawrence, 2003; Andersen et al., 2010; Santiago et al., 2012). It appears that improved nutrient availability induced higher nutrient uptake only at a rate proportional to the increase in growth rate, resulting in a dilution of the added nutrients by carbon and fairly stable foliar nutrient concentrations. Furthermore, *Pouteria* seedlings may store the added nutrients in stems or roots as investment in long-term survival. Such a strategy of late-successional species was reported by Raaimakers and Lambers (1996) for *Lecythis corrugata* seedlings in Guyana.

Biomass Allocation

The high root mass fraction of *Pouteria* seedlings compared to other shade-tolerant tropical tree seedlings (Burslem et al., 1995; Huante et al., 1995a; Wan Juliana et al., 2009) could be explained as an adaptation to generally low nutrient availability at the study site, as it was proven for our study site by the stimulation of tree root growth upon N and P (and also K) addition in the ingrowth core experiment of Graefe et al. (2010). These results indicate that trees put high priority on the maximization of belowground resource acquisition at this site as also reported by Paz (2003) and Poorter et al. (2012) for other tropical forest.

Upon nutrient addition we expected a compensatory change in carbon allocation to aboveground tissues as a result of increased soil nutrient availability (e.g., Marschner, 1995; Poorter et al., 2012), but surprisingly SMF was reduced and root:shoot ratio increased after P addition, and the root:leaf ratio was higher after N addition. In addition, LAR was not affected by nutrient addition. Enhanced C allocation to roots in a shade-tolerant species suggests that carbon and other elements are mainly stored in belowground organs to improve seedling survival under the very low light intensity at the forest floor of this stand (Raaimakers and Lambers, 1996).

CONCLUSIONS

Foliar nutrient concentrations are apparently not a good indicator of nutrient limitation in the rainforest tree *Pouteria torta*, since nutrient addition did change neither N or P concentration nor the ratio of both nutrients. The species' high abundance in the forests is another indication that it is well-adapted to the current growing conditions at this site. Nevertheless, the shade-tolerant species was capable of responding to improved nutrient supply with higher seedling growth rates, if the plants were able to survive in the relatively dark and nutrient-poor environment. On the population level, however, it is difficult to make predictions on how a future increase in nutrient deposition will change the recruitment success of this species, because individual growth increases are associated by increased herbivory and higher mortality. In any case, the moderate addition of 50 kg N ha^{-1} and 10 kg P ha^{-1} has provoked a number of morphological and physiological responses that likely will alter the performance of this common tree species and will have consequences for the competition with other tree species. In further studies it would be interesting to investigate if the co-occurring tree species respond similarly to N and P addition or if currently less abundant species gain competitive strength thus indicating potential changes in the composition of future forests under increased nutrient deposition.

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Nutrient-Induced Modifications of Wood Anatomical Traits of *Alchornea lojaensis* (Euphorbiaceae)

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Regarding woody plant responses on higher atmospheric inputs of the macronutrients nitrogen (N) and phosphorous (P) on tropical forests in the future, an adaptive modification of wood anatomical traits on the cellular level of woody plants is expected. As part of an interdisciplinary nutrient manipulation experiment (NUMEX) carried out in Southern Ecuador, we present here the first descriptive and quantitative wood anatomical analysis of the tropical evergreen tree species *Alchornea lojaensis* (Euphorbiaceae). We sampled branch wood of nine individual trees belonging to treatments with N fertilization, N+P fertilization, and a control group, respectively. Quantitative evaluations of eleven different vessel parameters were conducted. The results showed that this endemic tree species will be able to adapt well to the future effects of climate change and higher nutrient deposition. This was firstly implied by an increase in vessel diameter and consequently a higher theo. area-specific hydraulic conductivity with higher nutrient availability. Secondly, the percentage of small vessels (0–20 μm diameter) strongly increased with fertilization. Thirdly, the vessel arrangement (solitary vessels vs. multiple vessel groupings) changed toward a lower percentage of solitary vessel fraction (V_S), and concurrently toward a higher total vessel grouping index (V_G) and a higher mean group size of non-solitary vessels (V_M) after N and N+P addition. We conclude that higher nutrient availability of N and N+P triggered higher foliage amount and water demand, leading to higher cavitation risk in larger vessels. This is counteracted by a stronger grouping of vessels with smaller risk of cavitation to ensure water supply during drier periods that are expected to occur in higher frequency in the near future.

Keywords: branch wood, evergreen tropical montane forest, Euphorbiaceae, fertilization, quantitative wood anatomy

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INTRODUCTION

Besides water and light availability, temperature and salinity, the macronutrients nitrogen (N) and phosphorous (P) are considered as the most important factors controlling and limiting tree growth in the majority of the world's ecosystems (e.g., Cavelier et al., 2000; Hedin et al., 2009; Vitousek et al., 2010; Goldstein et al's., 2013). Since the beginning of the industrial age, both P and N cycles have skipped out of balance due to human activities and the use of fertilizer and fossil fuel (Smil, 2000; Wright, 2005; Galloway et al., 2008). Galloway et al. (2003) described the potential effect chains of increasing reactive N within the biogeochemical cycle, stressing that especially forests have a high

potential to accumulate and transform reactive N. In particular, tropical forests will sensitively react to expected higher N and P inputs, but the full extent of their responses is discussed controversially (e.g., Tanner et al., 1990; Homeier et al., 2012; Fisher et al., 2013; Powers et al., 2015).

Goldstein et al's. summarized (2013, p. 2) that after being released from nutrient limitation of N and P, trees will theoretically respond with higher growth rates. Additionally, the hydraulic properties can be oppositional influenced, leading to a higher xylem hydraulic conductivity, but not to a higher leaf hydraulic conductivity (efficiency of stem water supply per unit leaf surface area), although stomatal conductance will decrease to minimize water loss. Hence, it is assumed that higher nutrient availability results in a higher water deficit and this will consequently lead to more negative water potentials of fertilized trees.

These expectations result from the fact that secondary growth is directly controlled by the entire tree physiology, which in turn is compromised by external environmental factors (Fonti et al., 2010).

The vascular system (nonliving conductive cells in the xylem) of trees is responsible for the long distance water and nutrient transport from the roots to the leaves (Pittermann, 2010). However, the efficiency of the functional hydraulic properties is mainly compromised by the tradeoffs between conductivity and vulnerability to dysfunction, and has been described in detail by Tyree and Zimmermann (2002). Those tradeoffs are greatly influenced by conduit/vessel size and the arrangement and structure of the vessel network. For example, vessel grouping (min. two vessels are joined in radial or clustered groups) on the one hand increases the resilience to cavitation and might provide safety for the conductive system, but on the other hand the risk of hydraulic failure of individual vessel elements increases concurrently (Carlquist, 2001; von Arx et al., 2013). When analyzing the vascular system of branches one firstly needs to consider that trees alter their wood anatomical architecture along the axial pathway, affecting the hydraulic system from the root to the twigs differently (Tyree and Zimmermann, 2002; Sperry et al., 2008; McCulloh et al., 2010). Secondly, the wood structure of branches may differ from the stem's xylem structure, characterized by a denser, less elastic wood with a higher resistance to cavitation (Lachenbruch and McCulloh, 2014). And thirdly, tension wood is often present on the upper sides of branches which can profoundly affect the water transport due to its special kind of wood tissue, which is characterized by an internal gelatinous layer in fibers, a missing cell wall lignification, as well as fewer and smaller vessels (Barnett et al., 2014). These modifications need to be taken into account when analyzing hydraulic properties of branch wood since hydraulic functioning is controlled by mechanical demands (Fournier et al., 2014).

An increase in nutrient availability can directly influence cell formation and induce changes in vessel diameter size and arrangement (Zimmermann, 1978; Hacke, 2015).Consequently, tree species are expected to vary their hydraulic architecture due to environmental impacts, as described by Arnold and Mauseth (1999). However, studies focusing on nutrient-induced modification of wood anatomical traits are quite sparse (Harvey and Van den Driessche, 1997; Hacke et al., 2010; Hacke, 2015) and completely lacking in tropical regions at this time (Zuidema et al., 2013). But how can those changes of the vascular system be detected or calculated? Scholz et al. (2013) provided an overview of different methodological approaches how to quantify vessel or tracheid elements in plants. Numbers of different vessel parameters help comprehending possible adaptations or changes of the xylem structure. Accordingly, comparisons with e.g., the total growth rate, the production of biomass, the foliar morphology, and foliar chemistry can be drawn (Meinzer et al., 2008; Schuldt et al., 2013; Hoeber et al., 2014).

Our investigations of the vessel system were conducted on the evergreen tropical montane tree species *Alchornea lojaensis* Secco (Euphorbiaceae). This woody species of the Euphorbiaceae is endemic in Southern Ecuador. The wood is diffuse porous and vessels range from broader solitary to rather small to mid-scale grouped radial multiples. Thus, the species is well-adapted to humid habitats, where water stress is lacking and cavitation risks are naturally minimized (Carlquist, 2001). Further, the species shows a high adaptability to nutrient-poor soil conditions, resulting in high abundance within the study forest. Therefore, we expect a high susceptibility and sensitivity to increasing availability of N and P, reflected by changes in the vessel system.

The aim of this study was to investigate to what extent or direction the vascular system of *A. lojaensis* has been changed by an increased nutrient availability of N and P, and how this affects the species' theoretical hydraulic properties. Since the tree species has been poorly studied, we carried out a descriptive wood anatomy analysis based on thin sections and common microscopic techniques. Based on the quantitative parameter "vessel lumen area," we analyzed nutrient affected modifications of vessel diameter size, theo. area-specific hydraulic conductivity, vessel grouping indices, vessel packing functions and vulnerability index. Further, we point out possible consequences for the biodiversity and the species composition of the lower tropical montane forest in Southern Ecuador.

MATERIALS AND METHODS

Study Site

The study area Reserva San Francisco (RSF, 3°58′S, 79°04′W) covers an altitudinal range from 1.600–3.140 m a.s.l. at the northern slope of the Podocarpus National Park (PNP), located in the Cordillera Real, at the eastern escarpment of the Southern Ecuadorian Andes (Richter et al., 2013). This hotspot of biodiversity hosts more than 300 tree species of which the majority belongs to the plant families Lauraceae, Melastomataceae, and Rubiaceae (Homeier et al., 2010). Local climate is described by per-humid conditions with an average annual temperature of 15.3°C and an average annual precipitation of 2067 mm at 1950 m a.s.l. (Beck and Richter, 2008). Precipitation patterns are characterized by slight seasonality. Wettest conditions are predominant from May to August, while the driest months are October and November (Rollenbeck and Bendix, 2011). During this time, rainless periods of up to more than 2 weeks may occur, potentially leading to drought stress in this otherwise per-humid environment. The

dominant soils are acid cambisols with pH values of 3.8 in the A-horizon (Wilcke et al., 2002). However, in general, soils vary considerably within the study area and are mainly nutrient-poor. Total annual atmospheric nutrient depositions are estimated to reach 14–45 kg N ha^{-1} and 0.4–4.9 kg P ha^{-1}, respectively (Homeier et al., 2013).

Experimental Design and Branch Sample Design

Our study was carried out within an interdisciplinary, full-factorial nutrient manipulation experiment located inside the RSF at an altitude between 2020 and 2120 m a.s.l. (abbreviated "NUMEX," in the following, for details see Homeier et al., 2012). The fertilization experiment (**Figure 1**) was set up in a randomized, fourfold replicated block design, each consisting of four different treatments (N, P, N+P, and unfertilized control, 20 × 20 m plots per treatment). Nutrient applications started in 2008 and were repeated biannually. Total amounts of 50 kg ha^{-1} yr^{-1} of N (as urea), 10 kg ha^{-1} yr^{-1} of P (as $NaH_2PO_4 \cdot 2H_2O$) and the combination of both (N+P plots) were added on the respective plots (Homeier et al., 2013).

A. lojaensis Secco (Euphorbiaceae) is one of the most common tree species within the NUMEX plots, with an average annual stem diameter growth of 0.12 mm (unfertilized control, monitoring period: February 2008–January 2009, Homeier et al., 2012).

Forty-one species of the genus *Alchornea* are distributed along the pan-tropics, with a majority in Central and South America (Secco, 2008). However, *A. lojaensis* is one of the very few endemic species, being restricted to the Zamora-Chinchipe province (southern Ecuador) between elevations of 1900–2400 m a.s.l. (Homeier and Werner, 2008). Although, the evergreen broadleaved tree species *A. lojaensis* has been described morphologically by Secco (2008), neither its wood structure nor its wood anatomical parameters have been studied so far.

In February 2013, five years after the start of the fertilization experiment, we collected one branch sample per tree of nine *A. lojaensis* individuals within three different treatments of the NUMEX plots (Control, N and N+P). Within each treatment, three individual trees were sampled. In this study, specimens of P plots were not investigated due to technical difficulties regarding the thin-section preparation.

Xylem Anatomical Measurements

Wood anatomical measurements and hydraulic calculations were performed on cross-sections of the collected branch samples. Each branch specimen was dehydrated with increasing ethanol concentration series and rotihistol, embedded in paraffin and cut into thin-sections (10 μm) with an electronic rotation microtome (Leica, Germany). All transverse sections, cleaned of residual paraffin, were stained with a mixture of Fuchsine, Chrysoidin and Astra blue (FCA solution after Etzold, 2002) and finally fixed with Canada balsam.

Digital photos with a fourfold magnification (Leica, microscopic system) were taken and merged together to analyze the entire xylem area, assuming that the complete area is conductive. To simplify subsequent measurements, vessel lumen was manually edited by using Adobe Photoshop. While solitary vessels were colored red, grouped vessels remained white. We divided every cross-section into four parts (through the pith) to achieve faster measurement and to provide a better measurement accuracy.

Vessel Diameter and Hydraulic Calculations

WinCELL 2010a software (®Regents, Canada) was used to measure vessel lumen cross sectional area (VA, μm^2) of each single vessel. Vessel area was converted to an idealized arithmetic vessel diameter (d, μm) assuming circularity of vessels (Scholz et al., 2013). Based on each single arithmetic vessel diameter (d), we calculated the hydraulic vessel diameter (d_h, μm, Equation 1) according to Tyree and Zimmermann (2002) as:

$$d_h = \left(\frac{\sum d^4}{n}\right)^{\frac{1}{4}} \quad (1)$$

The theoretical hydraulic conductivity (k_h^{theo}, $m^4 MPa^{-1}$ s^{-1}) was evaluated from the vessel radii (r, μm) using the Hagen-Poiseuille law (Equation 2, modified after Tyree and Zimmermann, 2002), where η is the viscosity of water, calculated at 20.0°C.

$$k_h^{theo} = \frac{\pi \sum r^4}{8_\eta} \quad (2)$$

Subsequently, we determined the theo. area-specific hydraulic conductivity (k_s^{theo}, kg $m^{-1} MPa^{-1}$ s^{-1}, Equation 3) by including the density of water (ρ) at 20.0°C (Tanaka et al., 2001), and dividing it by the total xylem area (A_{xylem}).

$$k_s^{theo} = \frac{k_h^{theo} \rho}{A_{xylem}} \quad (3)$$

Vessel Frequency and Vulnerability Index

To determine vessel frequency (VF, average number of vessels per mm^2), 10 segments with an area of 1 mm^2 were randomly selected on each cross section and all vessels were counted as individuals (Wheeler, 1986). Based on the arithmetic vessel diameter (d) and the number of vessels per mm^2, we calculated the averaged area-weighted vessel diameter (d_a; μm) for each field by using Equation 4 and computed the maximum vessel packing limit, assuming square packing (McCulloh et al., 2010, 2011).

$$d_a = \left(\frac{\sum d^2}{n}\right)^{0,5} \quad (4)$$

Vessel frequency per mm^2 and area-weighted vessel diameter were also used to calculate the vessel vulnerability index (VI; Carlquist, 1977; **Table 2**). The ratio of these two variables offers a rough assessment about the trees' sensitivity to the risks of cavitation or embolism.

Vessel Arrangement and Vessel Indices

Table 1 provides an overview of three different indices that were defined to quantify vessel groupings. To achieve optimal results,

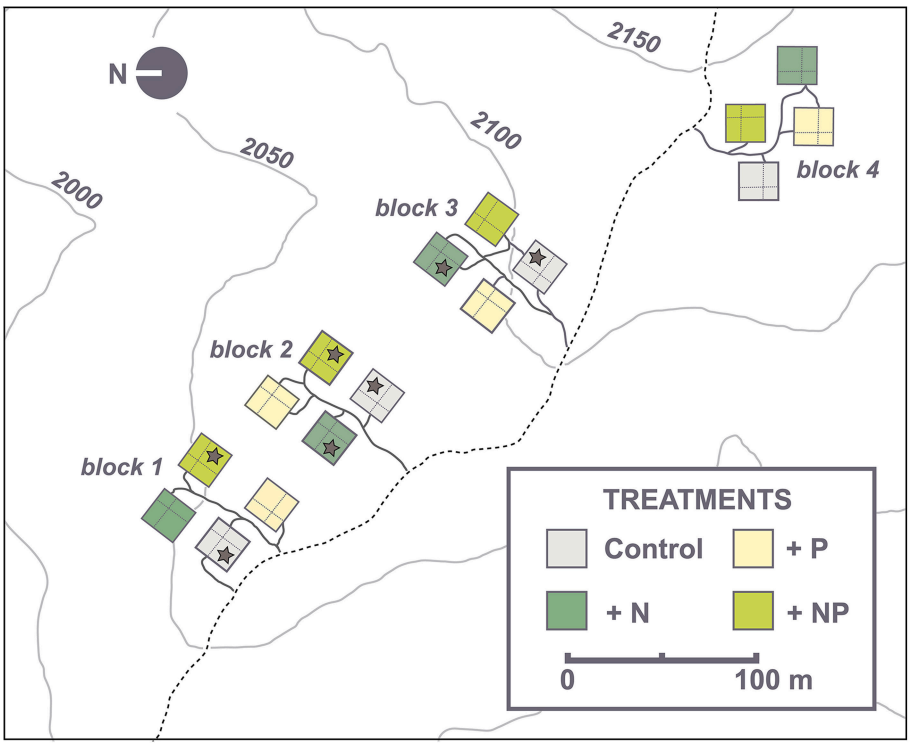

FIGURE 1 | NUMEX plot design. Control plots are colored in gray. Nitrogen plots (N) are colored in dark green. Phosphorous plots (P) are colored in light yellow and nitrogen plus phosphorous (NP) plots are colored in light green. Asterisks indicate the sample plots.

TABLE 1 | Index, ratio, definition, and references of different vessel grouping indices.

Index	Ratio	Definition	References
Vessel grouping index (V_G)	$\frac{N_{vessels}}{N_{groupings}}$	Mean number of vessels per group	Carlquist, 2001
Vessel solitary index (V_S)	$\frac{N_{solitary\ vessels}}{N_{vessels}}$	Ratio of solitary vessels to all vessels	Scholz et al., 2013; von Arx et al., 2013
Vessel multiple index (V_M)	$\frac{N_{multiple\ vessels}}{N_{multiple\ groupings}}$	Mean group size of non-solitary vessels	Scholz et al., 2013; von Arx et al., 2013

it was necessary to count every vessel separately, based on the total xylem area (Wheeler, 1986). Additionally, we evaluated the vessel frequency distribution (absolute and relative) of different vessel group sizes (von Arx et al., 2013).

Statistical Methods

All statistical analyses were performed with IBM® SPSS® Statistics Version 23. All calculations of the mean values were performed at the tree level [$n = 3$ (C): 3 (N): 3 (N+P)]. Requirements for normal distribution and statistical methods were checked with the Shapiro Wilk test and also visually (histogram). For confirming homogeneity of variance we used the Levene Test. In case of variance heterogeneity, significance threshold was increased to 0.01 (Bühl, 2008).

Treatment effects on different wood anatomical parameters (arithmetic vessel diameter, hydraulic vessel diameter, theo. area-specific hydraulic conductivity, vessel frequency, vulnerability index, and vessel indices) were conducted on the tree mean values and compared in a univariate ANOVA (factor 1 = treatment) with a Scheffé *post-hoc* test. Vessel arrangements effects (solitary vessels vs. multiple vessels) on arithmetic vessel diameter differentiated into treatments were tested with an unpaired student-*t*-test. To evaluate the influence of the combination of these two variables on vessel anatomical parameters we performed a multivariate analysis of variance (factor 1 = treatment; factor 2 = vessel arrangement) including η^2 effect size of the model. To show the relationship between vessel frequency and area-weighted diameter between the different treatments we performed a linear regression at the single tree level ($n = 10$ per tree; $n = 30$ per treatment). The corresponding means were compared at the tree level.

RESULTS

Descriptive Analysis of Wood Anatomical Traits of *Alchornea lojaensis*

Distinctness of growth ring boundaries (GRB, marked by arrows) dramatically differed along the whole branch xylem area. At several points, GRBs are clearly visible by flattened and thickened libriform fibers (LF, **Figures 2A,B**). However, a continuous, clear "tree ring" boundary could not be detected. Typically for

TABLE 2 | Mean percentage ratio between solitary vessels and multiple vessels, mean vulnerability index (VI), and mean vessel grouping indices for all three treatments.

Treatment		Percentage ratio				VI		Vessel grouping indices					
		Solitary vessel		Multiple vessels				V_G		V_M		V_S	
	n	*M*	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*
C	3	30.80	3.87	69.20	3.87	4.02	1.67	1.92	0.10	3.26	0.10	0.31	0.03
N	3	26.80	2.69	73.20	2.69	7.31	4.07	2.07	0.14	3.36	0.23	0.27	0.02
N+P	3	30.00	8.52	70.00	8.52	6.23	2.97	2.02	0.32	3.49	0.50	0.30	0.09

C, Control; N, Nitrogen; N+P, Nitrogen + Phosphorous; n, sample size; M, mean value; SD, standard deviation; VI, Vulnerability Index; VG, Vessel grouping index; VS, Vessel solitary index; VM, Vessel multiple index; Means were calculated at the tree level.

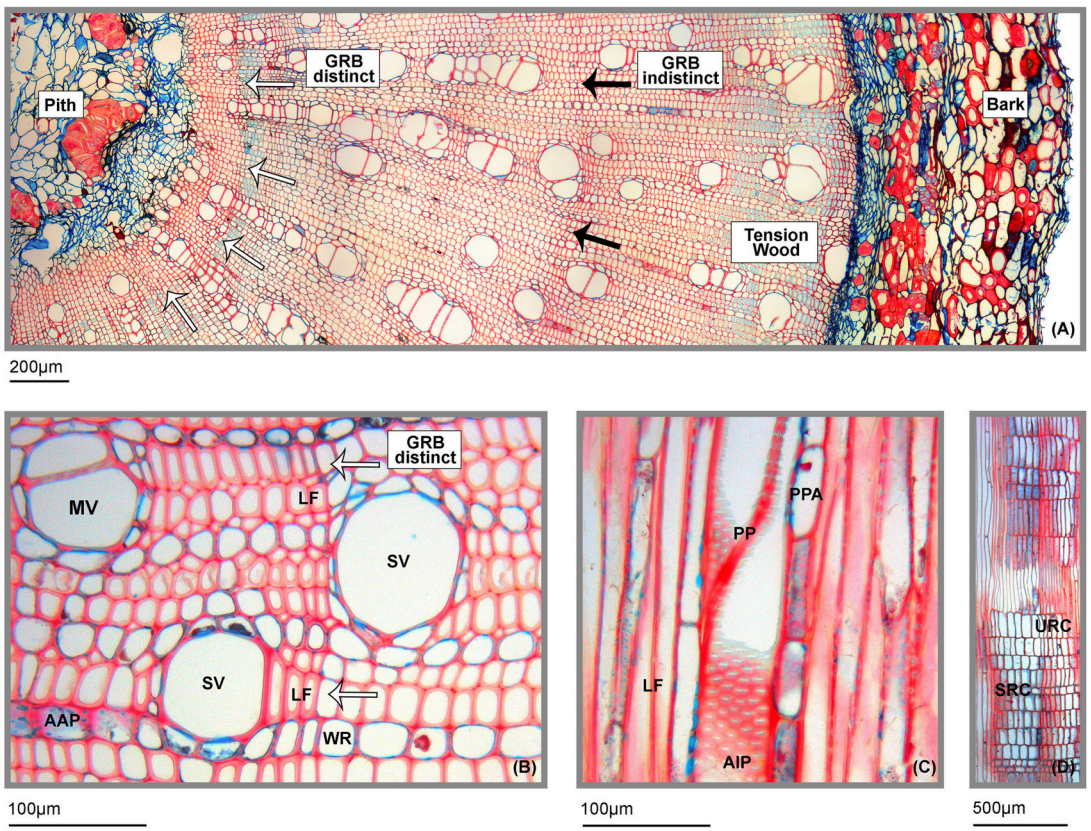

FIGURE 2 | Characteristics of wood anatomical traits of *A. lojaensis*. (A) Part of a cross section of *A. lojaensis* from the pith to the bark (10-fold magnification). Partly distinct growth ring boundaries (GRB) are indicated by white arrows; indistinct growth ring boundaries are indicated by black arrows. Tension wood within is the xylem area can be recognized by light blue dyed gelatinous fibers which mainly consist of cellulose and hemicellulose. **(B)** Close up view of a cross section of *A. lojaensis* (20-fold magnification). SV, solitary vessel; MV, multiple vessels; LF, flattened and thickened libriform fibers; WR, wood ray; AAP, axial apotracheal parenchyma; **(C)** Tangential section of *A. lojaensis* (20-fold magnification). LF, libriform fibers; PP, perforation plate; AIP, alternate positioned intervessel pits; PPA, paratracheal parenchyma; **(D)** Radial section of a wood ray of A. lojaensis (4-fold magnification). URC, upright rectangular ray cells; SRC, squared ray cells.

branch wood of dicots, we found several parts of tension wood within the xylem area which can be recognized by blue dyed gelatinous fibers whose cell walls mainly consist of cellulose and hemicellulose (**Figure 2A**).

The wood is diffuse-porous, consisting of both, one third of solitary vessels (SV, $M = 29.2\%$, $SD = 2.12$) and two third of radial multiple vessels (MV, $M = 70.8\%$; $SD = 2.12$). Most of the multiples comprise short rows consisting of 2–5 vessels; however, maximum row numbers can reach up to 10–15 vessels (**Figure 2A**). Vessel outlines are rounded with a scanty paratracheal parenchyma (PPA, **Figure 2C**). Intervessel pits are positioned alternately (AIP, **Figure 2C**); perforation plates (PP, **Figure 2C**) are simple and diffuse; apotracheal axial parenchyma (AAP, **Figure 2B**) is distinct. Wood stability is provided by non-septated libriform fibers (LF, **Figures 2A,B**). Vascular or vasicentric tracheids are absent. Wood rays (WR, **Figures 2B,D**)

are exclusively uniseriate and hetero-cellular, composed of upright rectangular (URC, **Figure 2D**) and squared ray cells (SRC, **Figure 2D**). Radial canals are occasionally present in rays.

Quantitative Analysis of Wood Anatomical Traits of *Alchornea lojaensis*

Arithmetic Vessel Diameter: Distribution and Means

The analyses of the percentage ratio between solitary vessels and multiple vessels obtained slightly diverging results for the individual treatments (**Table 2**), indicating a comparatively smaller proportion of solitary vessels after N addition compared to the C and N+P treatments. However, differences were not statistically significant [$F_{(2,\ 6)} = 0.424$, ρ = n.s].

Striking different frequency distribution patterns of vessel diameter classes (20 µm width) existed for total (**Figure 3A**), solitary (**Figure 3B**), and multiple vessels (**Figure 3C**). While solitary vessels were almost normally distributed, frequency of multiple vessels decreased linearly from small (20–40 µm) to large (180–200 µm) diameter classes. Considering exclusively solitary vessels, smaller arithmetic vessel diameters between 60 and 80 µm appeared most frequently for C and N+P, whereas highest frequency between 80 and 100 µm occurred after N-fertilization. Regarding the frequency distribution of

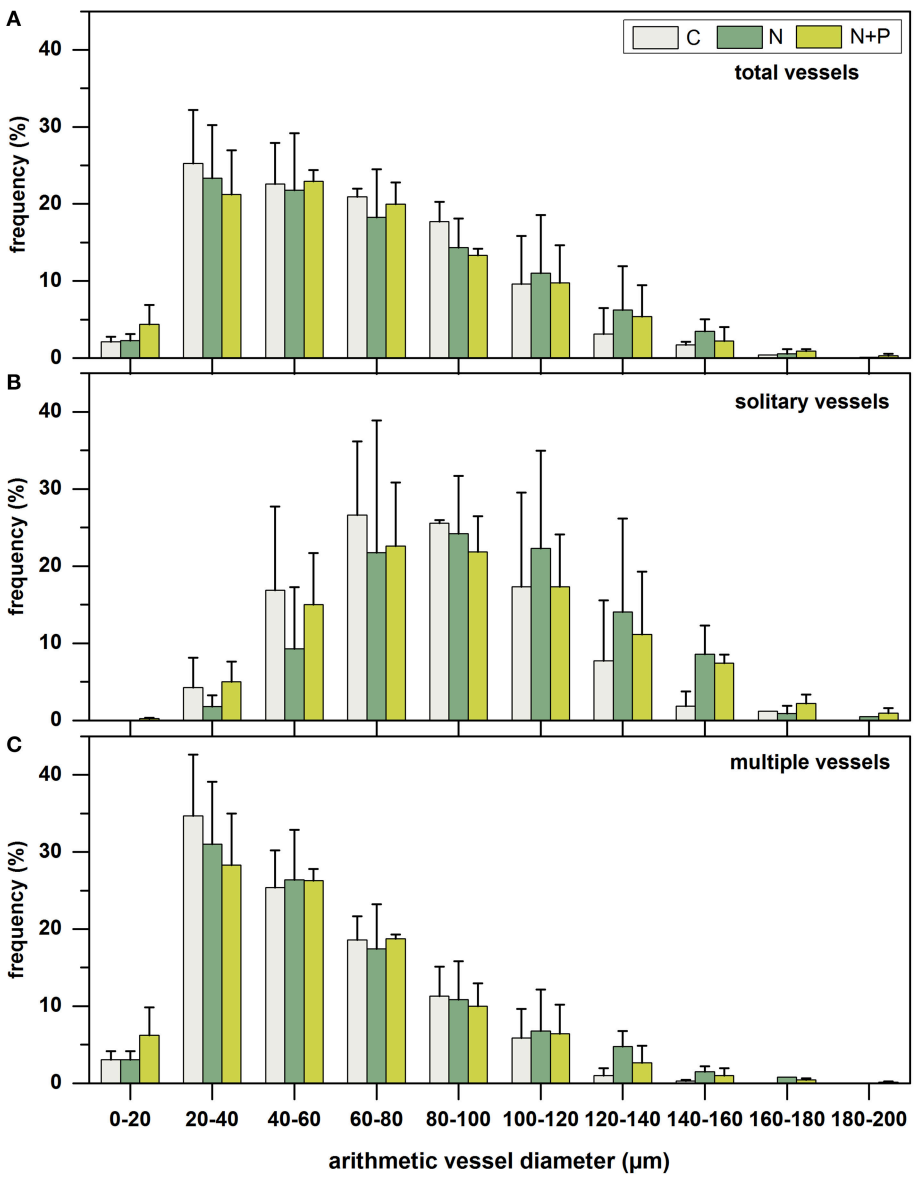

FIGURE 3 | Mean frequency distribution (%) of arithmetic vessel diameter classes (20 µm width) of total number of vessels (upper panel, A), solitary vessels (middle panel, B), and multiple vessels (lower panel, C), separated into control group (C = gray; *n* = 3), nitrogen fertilized group (*N* = dark green; *n* = 3), and nitrogen plus phosphorous fertilized group (N+P = light green; *n* = 3). Columns represent mean values of the three trees per treatment; whiskers represent the respective standard deviations.

multiple vessels, highest incidence could be found for small vessel diameters between 20 and 40 µm for all treatments. The frequency of the smallest diameter class between 0 and 20 µm clearly increased after N and N+P addition compared to the control samples.

Figure 4 illustrates that fertilization also slightly affected mean arithmetic vessel diameter size. For the case of all vessels, N-addition ($M = 68.16$, $SD = 12.91$) caused larger means than the combination of N+P ($M = 66.67$, $SD = 10.70$) or no fertilization ($M = 63.48$, $SD = 9.73$), although not statistically significant. A similar inter-treatment pattern was also seen for exclusively solitary vessels (control: $M = 83.14$, $SD = 14.69$; nitrogen $M = 94.94$, $SD = 17.07$; nitrogen + phosphorous $M = 88.93$, $SD = 14.15$) and multiple vessels (control: $M = 54.48$, $SD = 7.09$; nitrogen $M = 58.20$, $SD = 10.24$; nitrogen + phosphorous $M = 57.08$, $SD = 8.97$), respectively. Whereby, the effect of fertilization was higher for solitary vessels.

The intra-treatment comparison showed that vessel arrangement had a significant effect on vessel diameter size, which was expressed by significantly larger diameters of solitary vessels than of multiple vessels. (control [$t_{(4)} = 3.034$, $\rho < 0.05$], nitrogen [$t_{(4)} = 3.196$, $\rho < 0.05$], and nitrogen + phosphorous [$t_{(4)} = 3.293$, $\rho < 0.05$]).

The two-way ANOVA reaffirmed that treatment effect [factor 1, $F_{(2,\ 18)} = 0.0576$, $\rho =$ n.s., $\eta^2 = 0.008$) and the effect of vessel arrangement [factor 2, $F_{(1,\ 18)} = 30.076$, $\rho < 0.001$, $\eta^2 = 0.715$] on vessel diameter size, whereby the latter had a substantially greater effect. An interaction between these two factors could not be determined [$F_{(2,\ 18)} = 0.158$, $\rho =$ n.s., $\eta^2 = 0.026$].

Hydraulic Vessel Diameter and Theoretical Area-Specific Hydraulic Conductivity (k_s^{theo})

Inter-treatment comparison showed slightly different patterns of the mean hydraulic vessel diameter (**Table 3**), indicating larger diameters after fertilization. Consequently, N and N+P fertilization led to an increasing k_s^{theo} for all vessels, for solitary vessels as well as for multiple vessels. However, k_s^{theo} conductivity was highest for the N+P samples. Astoundingly, multiple vessels of the N+P group ($0.89 \cdot 10^{-2}$ $m^2\,MPa^{-1}\,s^{-1}$) reached similar k_s^{theo} like solitary vessels of the control group ($0.92 \cdot 10^{-2}$ $m^2\,MPa^{-1}\,s^{-1}$), indicating that the high number of small diameter multiple vessels (N+P) can compensate for susceptibility to extreme climatic events like droughts. This also means that the high k_s^{theo} of total vessels for all treatments was a result of the high number of smaller vessels.

Vessel Frequency and Vulnerability Index

An apparent, significant converse relationship between area-weighted vessel diameter and vessel frequency was found for every single treatment (**Figure 5**), basically signifying that the larger the area-weighted diameter of a vessel, the fewer number of vessels could fit into 1 mm^2 ($p < 0.5$). However, the slope of the regression functions was steeper for the control treatment than for the fertilized treatments.

Mean area-weighted diameters were larger after fertilization compared to the control (**Figure 5**), but, although mean area-weighted diameters after N and N+P addition were nearly the same, fewer vessels were packed in a given area after fertilization

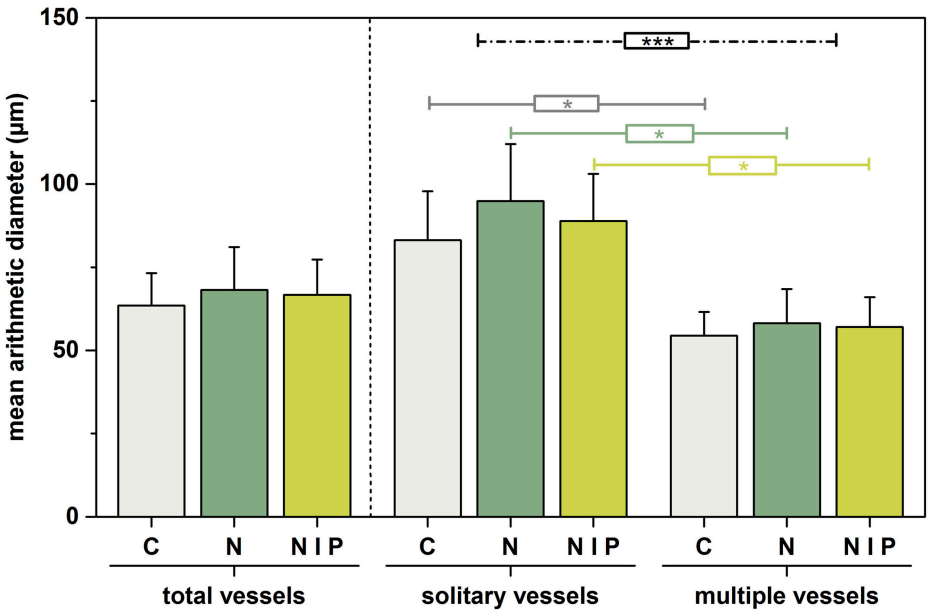

FIGURE 4 | Alteration of mean arithmetic vessel diameter size in response to fertilization for the total number of vessels (left panel) and for solitary and multiple vessels (middle and right panel). Columns represent mean values of the three trees per treatment; whiskers represent the respective standard deviations. Corresponding colored lines mark significant inter-treatment comparisons due to vessel arrangement (Student –*t*-test). Black line marks significant difference between vessel arrangements as a result of Two-way ANOVA. Significance levels are marked by $^*p < 0.05$, $^{***}p < 0.001$. C, control, gray; $n = 3$; *N* = nitrogen, dark green, $n = 3$; N+P = nitrogen + phosphorous, light green, $n = 3$.

TABLE 3 | Mean hydraulic vessel diameter (μm) and theo. area-specific hydraulic conductivity [k_s^{theo} (m^2 MPa^{-1} $s^{-1}10^{-2}$)] differentiated into vessel arrangement and treatment.

Vessel arrangement	Treatment		Hydraulic vessel diameter (μm)		Theo. area-specific hydraulic conductivity [k_s^{theo} (m^2 Mpa^{-1} $s^{-1}10^{-2}$)]	
		n	*M*	*SD*	*M*	*SD*
Total vessel	C	3	79.27	11.67	1.63	0.28
	N	3	85.93	17.41	1.73	0.79
	N+P	3	86.60	14.58	1.98	0.43
Solitary vessels	C	3	92.32	14.59	0.92	0.19
	N	3	103.20	18.58	0.97	0.43
	N+P	3	101.77	17.25	1.10	0.21
Multiple vessels	C	3	70.05	8.92	0.71	0.26
	N	3	75.67	16.01	0.76	0.36
	N+P	3	76.53	13.29	0.89	0.44

C, Control; N, Nitrogen; N+P, Nitrogen + Phosphorous; n, sample size; M, mean value; SD, standard deviation; Means were calculated at the tree level.

with N and caused the highest concomitant reduction of vessel frequency.

These results were strengthened by the vulnerability index. VI values varied considerably between treatments (**Table 2**) although not statically significant. After fertilization, vessel diameters were larger than those of the control group, but also showed a considerable increase of the vulnerability index, causing a higher susceptibility to environmental changes.

Vessel Indices and Vessel Group Sizes

Insignificant differences in vessel indices were determined between the treatments (**Table 2**). However, after fertilization, V_G and V_M tended to increase while the control samples showed highest proportions of solitary vessels (V_S). That apparent relationship suggested that nutrient addition caused more grouped, but fewer and larger solitary vessels. Unfortunately, due to the relative small sample size and resulting low degrees of freedom, correlations could not be verified statistically.

Relative frequency of different group sizes also varied between treatments. **Table 4** shows the absolute numbers of the vessel group sizes for each sample as well as the percentage mean values at the tree level with the respective standard deviation. In all treatments, more than 50% of the vessel groupings consisted of solitary groupings, showing the smallest proportion for after N fertilization. However, N fertilization affected group sizes between 2 and 6 vessels, whereas the combination of N+P resulted in the development of groupings with more vessel elements.

DISCUSSION

Descriptive Analysis of Wood Anatomical Traits of *Alchornea lojaensis*

Woody species of Euphorbiaceae show vessel arrangements ranging from solitary to multiple vessels in double-digit range and present libriform fibers as imperforate tracheary elements (Carlquist, 1984). By counting each vessel individually, we could affirm this pattern for *A. lojaensis*, with about 31% solitary vessels and 69% multiple vessels for the unfertilized trees (**Table 2**). Strong visual similarities in wood structure were found with *A. grandiflora* and *A. pearcei* from Brazil (Chavarri and Léon, 2005).

Contrary to the evergreen species *A. sidifolia* from Brazil (Filho et al., 2004), we could not detect distinct increment zones in our studied branch samples of *A. lojaensis* (**Figures 2A–D**). Although flattened and thickened libriform fibers were detected for both species, a distinct continuous band of radially flattened and thickened fibers marking a growth boundary was not found in branches of *A. lojaensis*. The same applies to the closely related species *A. triplinervia,* also occurring within the study area and in South Brazil (Homeier and Werner, 2008). The different wood anatomical properties might be explained by different growth strategies related to climatic differences between the two ecosystems (Worbes, 1995). While a distinct dry period between June and August with less than 60 mm precipitation per month might induce cambial dormancy accompanied by anatomically visible increment zones of the Brazilian species (Filho et al., 2004), *A. lojaensis* is well-supplied by soil water the whole yearlong. Therefore, the whole xylem area of the branches is assumed to be completely conductive. This assumption was further supported by the fact that tyloses were not observed.

Arithmetic Vessel Diameter: Distribution and Means

In order to assess the full extent of vessel anatomy variability of *A. lojaensis* affected by nutritional impacts, we compared the results for the control group with a study from Schuldt (2005), which was carried out within the same research area (PNP, without fertilization). In that study, mean vessel diameter of different twigs measured 41.04 μm at an elevation of 1890 m a.s.l. In our study, *A. lojaensis* reached 155% larger mean vessel diameter ($M = 63.48$, $SD = 9.73$) at almost the same elevation (**Figure 4**). However, one needs to consider the generally decreasing size of vessels from stems to twigs (Tyree and Zimmermann, 2002). Nevertheless, those values can still be classified as vessels with rather small to average diameter size (Wheeler et al., 1989).

N and N+P fertilization triggered changes in vessel size, with a tendency toward larger diameters (**Figure 4**). This confirms observations for hybrid poplar, where an increase of N and/or P availability was reported to enhance vessel diameter in stems (Harvey and Van den Driessche, 1997; Hacke et al., 2010; Hacke, 2015). However, inverse responses of vessel diameter size after nutrient addition were also found (e.g., Arnold and Mauseth, 1999; Faustino et al., 2013). Such diverging fertilization effects resulted mostly from different elevations, soil types, or tree species.

Besides treatment effects, the vessel arrangement of *A. lojaensis* revealed a high impact on vessel diameter size, which is consistent with findings of Arnold and Mauseth (1999) for *Cereus peruvianus* and *Cereus tetragonus*. Hence, this effect is important to be considered when analyzing diffuse porous

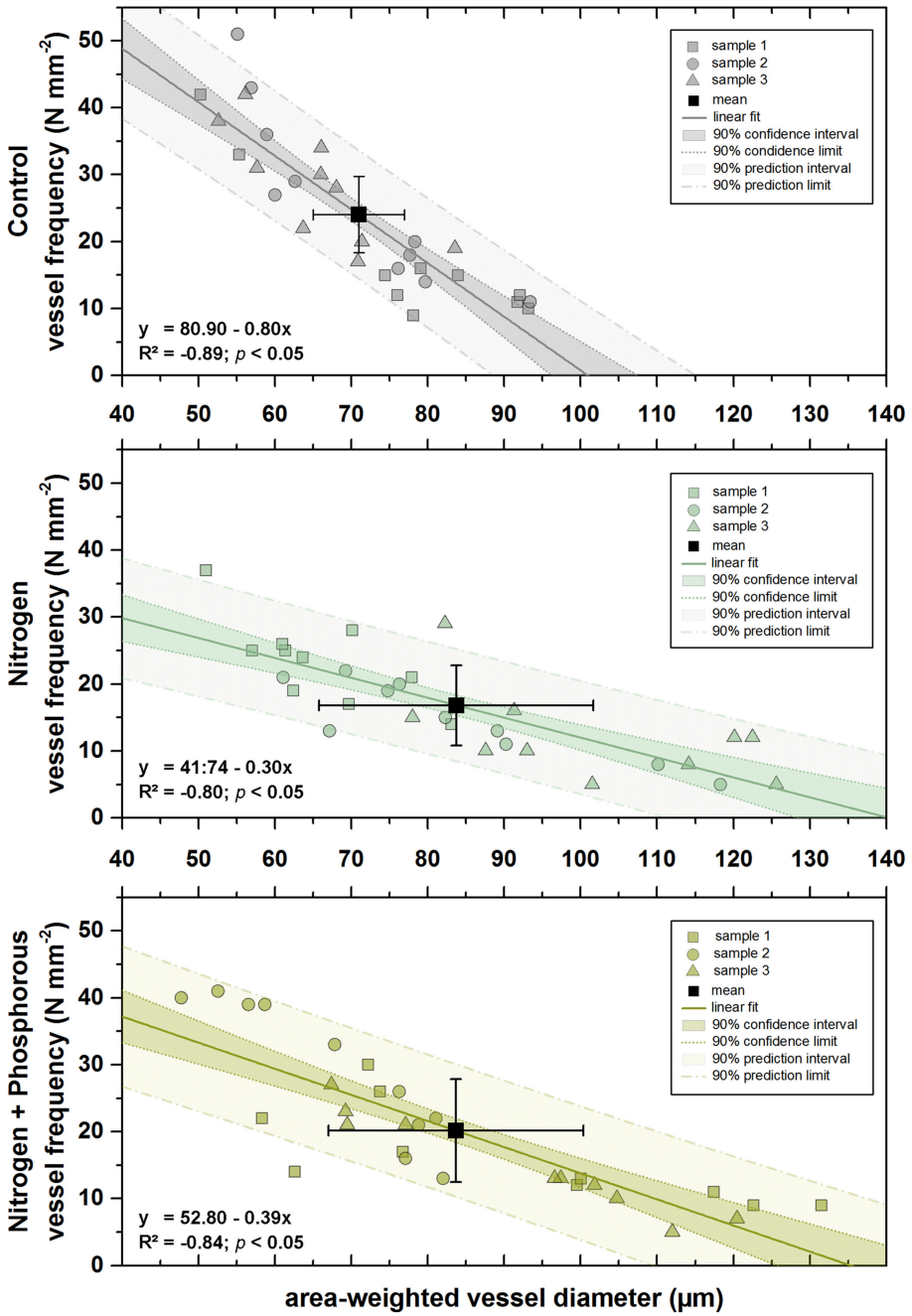

FIGURE 5 | Regression models between area-weighted diameter (µm) and vessel frequency (N mm^{-2}) of different fertilization treatments. Upper panel = control treatment; values of sample 1, 2, and 3 are marked by gray rectangles, circles, and triangles, respectively ($n = 10$ in each case). Mean value of all samples ($n = 3$) is marked by the black rectangle ($n = 10$), respective standard deviations are marked by black whiskers. Regression model: gray continues line indicates the linear fit, gray dotted lines indicate 90% confidence limits, gray dot-dashed lines indicate 90% prediction limits. **Middle panel** = nitrogen treatment; values of sample 1, 2, and 3 are marked by dark green rectangles, circles, and triangles, respectively ($n = 10$ in each case). Mean value of all samples ($n = 3$) is marked by the black rectangle ($n = 10$), respective standard deviations are marked by black whiskers. Regression model: dark green continues line indicates the linear fit, dark green dotted lines indicate 90% confidence limits, dark green dot-dashed lines indicate 90% prediction limits. **Lower panel** = nitrogen + phosphorous treatment; values of sample 1, 2, and 3 are marked by light green rectangles, circles, and triangles, respectively ($n = 10$ in each case). Mean value of all samples ($n = 3$) is marked by the black rectangle ($n = 10$), respective standard deviations are marked by black whiskers. Regression model: light green continues line indicates the linear fit, light green dotted lines indicate 90% confidence limits, light green dot-dashed lines indicate 90% prediction limits. Respective regression equations with significance values are shown in each panel, respectively.

TABLE 4 | Absolute numbers and mean relative frequency distribution of vessel group size.

Treatment			Vessel group size															Total number of groupings
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
C	Sample 1	n	594	152	105	59	24	13	6	3	4	–	–	1	–	–	–	961
	Sample 2	n	215	82	40	30	18	7	8	1	–	–	–	–	–	–	–	401
	Sample 3	n	561	152	87	45	24	14	11	3	–	4	3	1	2	1	–	908
	M	%	59.07	17.63	10.17	6.20	3.20	1.53	1.27	0.27	0.13	0.13	0.10	0.07	0.07	0.03	0.00	
	SD		4.73	2.44	0.67	1.25	1.13	0.15	0.70	0.06	0.23	0.23	0.17	0.06	0.12	0.06	0.00	
N	Sample 1	n	286	92	55	45	24	17	6	6	3	3	2	–	–	–	–	539
	Sample 2	n	448	151	89	55	29	14	7	4	–	–	1	–	–	1	–	799
	Sample 3	n	430	134	105	47	31	18	5	1	1	–	1	–	–	–	–	733
	M	%	54.93	17.77	11.63	7.10	4.03	2.43	0.87	0.57	0.27	0.20	0.20	0.00	0.00	0.03	0.00	
	SD		1.61	0.99	1.76	1.11	0.45	0.71	0.25	0.50	0.29	0.35	0.17	0.00	0.00	0.06	0.00	
N+P	Sample 1	n	363	136	98	60	42	18	12	6	2	4	1	1	3	-	1	746
	Sample 2	n	532	74	96	48	31	19	12	8	1	5	1	1	–	2	1	831
	Sample 3	n	415	125	44	35	7	9	3	4	1	1	–	–	–	–	–	644
	M	%	59.03	15.50	10.50	6.40	3.47	2.03	1.17	0.80	0.20	0.43	0.07	0.07	0.10	0.07	0.07	
	SD		8.95	5.75	3.29	1.40	2.26	0.55	0.59	0.20	0.10	0.21	0.06	0.06	0.17	0.12	0.06	

Absolute numbers are based on the single tree level. Mean values (in %) are based on the mean tree level (n = 3:3:3). Total number of groupings is calculated by the sum of all groupings, based on the tree level. C, Control; N, Nitrogen; N+P, Nitrogen + Phosphorous; n, absolute number of groupings; M, mean.

wood, since potential fertilization effects might be masked when vessels of different grouping size are analyzed together. Only by analyzing multiple and solitary vessels separately, the full effect of fertilization on vessel arrangement will become apparent, likewise we could find a slightly stronger influence on solitary vessels.

Theoretical Area-Specific Hydraulic Conductivity (k_s^{theo})

Changes in vessel diameter size generally provide inferences about the potential radial growth rates of trees, since larger vessels have a higher k_s^{theo} and consequently ensure increased water supply to support enhanced transpiration and photosynthetic rates (e.g., Meinzer et al., 2008, 2010; Smith et al., 2013; Hoeber et al., 2014; Oladi et al., 2014). The relation between higher growth capacity and vessel size becomes understandable, considering that the theo. hydraulic conductivity is related to the fourth power of the vessel radius (Tyree and Zimmermann, 2002). For *A. lojaensis* we calculated a high k_s^{theo} of $1.63 \cdot 10^{-2}$ $m^2\,MPa^{-1}\,s^{-1}$ at 20.0°C at the control site (**Table 3**). However, twigs examined by Schuldt (2005) reached computed theo. values of $2.2 \cdot 10^{-2}$ $m^2\,MPa^{-1}\,s^{-1}$ within the same study area. Both results can be classified as rather high values k_s^{theo} which appears to have been due to the fact, that all calculations were based on idealized vessel diameters and do not correspond to physically ideal circular capillaries.

Fewer but larger vessels are regarded to be more efficient in water and nutrient transport than more, but narrow vessels (Zanne et al., 2010). Therefore, due to N and N+P fertilization, k_s^{theo} of *A. lojaensis* was expected to increase to the same extent, as it was the case for arithmetic vessel diameter (Tyree and Zimmermann, 2002). However, k_s^{theo} tended to increase most strongly after N+P fertilization, most probably resulting from higher V_m (**Table 2**) compared to N fertilization.

Vessel Frequency, Vulnerability Index, and Vessel Grouping

Coincidently with increasing area-weighted vessel diameter, vessel frequency decreased. This inverse relationship is often found in wood anatomical studies (e.g., Zimmermann, 1978; Tyree and Zimmermann, 2002; McCulloh et al., 2010, 2011; Schuldt et al., 2013). Although the area-weighted vessel diameter might not reflect real measurements, N ($M = 83.74$, $SD = 17.95$) and N+P ($M = 83.67$, $SD = 16.68$) fertilized samples had nearly the same mean area-weighted vessel diameters (**Figure 5**). However, the combination of both nutrients ($M = 20.17$, $SD = 7.67$) lead to about 20% more vessels in the xylem than after N addition ($M = 16.83$, $SD = 5.99$). Thus, *A. lojaensis* probably compensates smaller solitary vessel diameters by a higher packing of grouped vessels after N+P addition.

The relationship between vessel frequency, vulnerability, resistance to cavitation, and hydraulic conductivity is relatively well-understood and several studies examined the trade-off between efficiency and safety (e.g., Hacke et al., 2006; Sperry et al., 2008; Zach et al., 2010). At first glance, the quite high vulnerability index of fertilized *A. lojaensis* samples suggests that this species is highly susceptible for environmental risks (**Table 2**). However, branch wood is typically denser than stem wood, and vessels are often reduced and narrower due to tension wood, resulting in higher embolism resistance (Lachenbruch and

McCulloh, 2014). This is substantiated by our investigations on the drought sensitivity of *A. lojaensis* using dendrometers (Homeier et al., 2013; Spannl et al., 2013). Despite several dry spells of four to nine consecutive rainless days, the daily amplitude of stem diameters showed no significant difference between fertilized and non-fertilized *A. lojaensis* tree individuals. Carlquist (1977, 2001) postulated that vessel groupings, in particular radial multiples, provide safety against cavitation by taking over the function of earlier formed vessels without alteration of the conductive system. For instance, if a vessel within a group is blocked by embolism, the other remaining vessels can maintain the axial water transport, thus decreasing drought vulnerability.

A higher degree of vessel grouping can be attained by a decrease in solitary vessels and larger groups of vessel multiples (von Arx et al., 2013). Confirming that hypothesis, we determined a decrease in V_S and an increase in V_G and V_M after addition of N and N+P in *A. lojaensis*. Hence, other vessel anatomical traits like, e.g., intervessel pit membrane structure or perforation pits are possibly responsible to provide more safety in the hydraulic system of angiosperms and also conifers (Tyree and Zimmermann, 2002; Cochard et al., 2009; Smith et al., 2013). Overall, despite the high vulnerability values, the risk of hydraulic failure for *A. lojaensis* at our study site can be deemed as rather small, because the per-humid conditions considerably minimize water stress, and risks of freezing or excessive heating are not given. However, these results have to be assessed with care due to the fact that the vulnerability Index was based on the diameter and not measured by a (t/b) ratio (t = wall thickness, b = lumen diameter, Hacke, 2015).

Wood Anatomy and Physiological Functioning

In tropical and subtropical forests, physiological functioning and stem growth are closely related to hydraulic and anatomical properties of the wood as well as to foliar chemistry and leaf morphological traits (e.g., Fichtler and Worbes, 2012, for a summary; Mayor et al., 2014). Homeier et al. (2012, 2013) examined total growth rates and leaf morphology of fertilized and non-fertilized *A. lojaensis* within the same study plots. After one year of fertilization (February 2008–January 2009), increasing stem diameter increment rates were already detected after N (+33%) and N+P fertilization (+42%, Homeier et al., 2012). However, increment rates were higher after the fifth year (N: +56%; N+P: +95%, Homeier, personal communication). Thus, not exclusively the vessel diameter, but also the k_s^{theo} might predict a potential maximal diameter growth rates (Fan et al., 2012). Specific leaf area and foliar N concentration of *A. lojaensis* increased after adding N and N+P (Homeier et al., 2012), indicating thinner but larger and less long-lived leaves (Poorter et al., 2009). Since foliar N concentration and photosynthetic capacity are directly related, fertilization is expected to also increase photosynthetic rates and related water demand (Wright et al., 2004; Homeier et al., 2012). As a consequence, increasing nutrient availability can to trigger more negative water potentials under drought stress (Goldstein et al's., 2013) and hence to a higher risk of vessel cavitation.

CONCLUSION

In this study, alterations of wood anatomical patterns of *A. lojaensis* induced by fertilization effects were detected. After the fertilization with N and N+P, increasing changes in arithmetic and hydraulic vessel diameter, k_s^{theo}, mean group size of non-solitary vessels and vulnerability index were found. Concomitantly, we found a decrease in vessel frequency as well as a lower V_S and an increasing V_G, triggered by higher nutrient availability. Our findings agree with Goldstein et al's. (2013) postulate about the effects of the macronutrients N and P on forest ecosystems. Accordingly, even moderate depositions of airborne N and P can cause striking changes in the structural features of tropical montane forests. We are not able to classify the main limiting nutrient to this tree species and ecosystem, since effects of P fertilization were not analyzed specifically.

However, we were able to detect that the effects of N and N+P are species specific. Tree species less common within the study area mostly "profited" from additional nutrients (Spannl et al., 2013) whereas more abundant species reduced their growth rates at the same time. *A. lojaensis* seems to be able to adapt well to expected effects of climate change in the study area due to major modifications of the vascular system. Therefore, it is expected that this tree species will become more abundant in this ecosystem in South Ecuador under future changing climatic conditions. However, to understand the complexity of the nutrient cycles of N and P and the responses of tropical forests, more long-term experiments like this study in Southern Ecuador are needed.

AUTHOR CONTRIBUTIONS

SS analyzed the data, designed the study, and wrote the first version of the manuscript and created graphics and tables. JH designed the NUMEX Experiment and collected the branch samples. AB and JH contributed in designing the experiment, writing of the manuscript, and interpretation of data and results.

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Detecting Terrestrial Nutrient Limitation: A Global Meta-Analysis of Foliar Nutrient Concentrations after Fertilization

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Examining foliar nutrient concentrations after fertilization provides an alternative method for detecting nutrient limitation of ecosystems, which is logistically simpler to measure than biomass change. We present a meta-analysis of response ratios of foliar nitrogen and phosphorus (RR_N, RR_P) after addition of fertilizer of nitrogen (N), phosphorus (P), or the two elements in combination, in relation to climate, ecosystem type, life form, family, and methodological factors. Results support other meta-analyses using biomass, and demonstrate there is strong evidence for nutrient limitation in natural communities. However, because N fertilization experiments greatly outnumber P fertilization trials, it is difficult to discern the absolute importance of N vs. P vs. co-limitation across ecosystems. Despite these caveats, it is striking that results did not follow "conventional wisdom" that temperate ecosystems are N-limited and tropical ones are P-limited. In addition, the use of ratios of N-to-P rather than response ratios also are a useful index of nutrient limitation, but due to large overlap in values, there are unlikely to be universal cutoff values for delimiting N vs. P limitation. Differences in RR_N and RR_P were most significant across ecosystem types, plant families, life forms, and between competitive environments, but not across climatic variables.

Keywords: leaf nutrients, N:P ratio, nutrient availability, nutrient limitation, response ratio, stoichiometry

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INTRODUCTION

Soil nutrient availability is a factor that defines the structure, function, and dynamics of many terrestrial ecosystems, particularly in those where nutrients are limited. The primary experimental approach used to determine soil nutrient limitation has been fertilization experiments (Tanner et al., 1998; Sullivan et al., 2014). At a species level, and from an evolutionary perspective, fertilization experiments seek to examine the relative importance of intra- and interspecific competition for abiotic resources. At ecosystem and global levels, fertilization experiments confirm biogeographic patterns of nutrient limitation, relating patterns to climate, substrate age, and other physical factors (Crews et al., 1995; Laliberté et al., 2012).

Most fertilization studies focus on plant biomass as the response variable, with increased growth after fertilization defining limitation (Eviner et al., 2000; Sullivan et al., 2014). However, foliar nutrient concentrations are another valuable way to assess nutrient limitation because numerous studies demonstrate that they reflect soil nutrient concentrations at spatial scales of single sites

(Shaver and Melillo, 1984; Valentine and Allen, 1990) and across chronosequences, environmental gradients, and ecosystem types (Vitousek, 1998; Han et al., 2005; Parfitt et al., 2005; Townsend et al., 2007; Ordoñez et al., 2009; Cleveland et al., 2011). When foliar nutrients are assessed as one-time measurements it is difficult to use them as evidence of nutrient limitation, because of the tremendous variation among study sites and the lack of universal cutoff values that indicate limitation. However, when foliar nutrients are assessed in relation to a fertilization event, they represent an alternative window on nutrient limitation (Güsewell and Koerselman, 2002; Wardle et al., 2004; Ågren, 2008; Sullivan et al., 2014). It is often easier and faster to collect foliar nutrient data before and after fertilization that it is to measure changes in biomass, plant height, or other size characteristics (Koerselman and Meuleman, 1996).

We present a meta-analysis of the fertilization studies that have measured foliar nutrient concentrations, to develop insights about nutrient limitation. In this review we limit our conclusions to proximate nutrient limitation (sensu Vitousek et al., 2010), in which fertilization is performed to measure the response of a biological process, rather than ultimate nutrient limitation where nutrient addition leads to whole-scale ecosystem change, such as species replacement. Meta-analysis is a tool that allows for detection of broad-scale patterns, not only through the inclusion of multiple studies, but through statistical techniques that account for differences in power and sample size among studies (Rosenberg et al., 2000). Recent examples of meta-analysis have examined plant biomass responses to fertilization (e.g., Elser et al., 2007; Vadeboncoeur, 2010; Harpole et al., 2011) but none to date have taken advantage of the large data sets available on foliar nutrient concentrations. We concentrate on nitrogen (N) and phosphorus (P) because they are the two most limiting nutrients to plants, and large amounts of foliar N and P data exist in the ecological literature. We present data either as: (1) response ratios (ratio of foliar nutrients in fertilized treatment to control treatment) or (2) ratios of nitrogen-to-phosphorus (hereafter N:P). Response ratios are a tractable way to compare nutrient responses, consistent with other meta-analyses on nutrient limitation (Elser et al., 2007). Because the response ratio index is a relative measure, it allows for cross-study comparisons that employ different methodologies and experimental designs (e.g., greenhouse vs. field studies), and additionally it was found to have great capabilities in detecting truly significant effects (Lajeunesse and Forbes, 2003). N:P values are included because foliar N:P appears to be correlated with N:P supply in both aquatic and terrestrial ecosystems and it has been suggested that specific N:P values isolate the type of limitation (i.e., N limitation, P limitation, or co-limitation; Redfield, 1958; Koerselman and Meuleman, 1996; Sterner and Elser, 2002; Tessier and Raynal, 2003; Güsewell, 2004). These ratios are prevalent in stoichiometric theory, which predicts that organisms have ideal proportions of chemical elements based on their metabolic requirements (Elser et al., 1996; Sterner and Elser, 2002; Ågren, 2008), that is consistent among organisms ranging from marine phytoplankton, terrestrial plants, and insect herbivores (Broadley et al., 2004; Güsewell, 2004; Karpinets et al., 2006).

We hypothesize that response ratios will differ in magnitude for N and P, given differences in their plant use and bioavailability. We base our hypothesis on the fact that plants have separate adaptations for maximizing N and P uptake (Wassen et al., 2005; Lambers et al., 2008; Hayes et al., 2014), and on studies that have suggested differential plant use of soil N and P when assimilated into leaf tissue (Güsewell and Koerselman, 2002; Townsend et al., 2007; Ostertag, 2010). Potential explanations could relate to the ability of plants to store P more effectively than N (Ostertag, 2010) or the inability to downregulate P uptake under high P supply. The latter mechanism has been supported by experimentally demonstrating P toxicity after P addition, for species from P-poor environments (Musick, 1978; Shane et al., 2004; Lambers and Shane, 2007; Standish et al., 2007; de Campos et al., 2013). We further hypothesize that any differences in response ratios between the two elements and within a given nutrient can be explained by: (1) climate, ecosystem type, and the competitive environment of the study (whether plants were fertilized as individuals, under intraspecific competition, or under interspecific competition), and (2) variables relating to species characteristics such as life form and family. Understanding the causes of variability in foliar nutrient concentrations is of primary ecological importance because leaf nutrients can predict resource capturing attributes, whole-plant functions, net primary productivity, and rates of nutrient cycling (Wright et al., 2005).

METHODS

Studies pertaining to foliar N and P response to fertilization were identified by searching combinations of the following key words: foliar, nitrogen, phosphorus, leaf, litter, fertilization, foliage, and fertilizer, in titles and abstracts using the Biological Abstracts search engine. Articles published up to the year 2011 were included in this meta-analysis. The search procedure resulted in 201 articles that contained relevant data. Only fertilization experiment studies were included (as opposed to natural fertility gradients) and studies needed to report foliar nutrient concentrations under both fertilized and unfertilized (control) conditions. The fertilization studies could have experimentally fertilized with N, P, and/or N and P. We did not classify fertilization methods beyond the nutrient added for several reasons. While we initially wanted to contrast organic vs. inorganic fertilizations, almost all studies added fertilizer in an inorganic form, making a comparison untenable. Studies diverged widely in the rate and timing of application, in ways that were often specifically chosen to complement ecosystem characteristics, but that made it difficult to develop categorical variables across studies. Many studies had multiple dosages of fertilizer applied and we addressed this methodological issue by including only the entries responding to the highest dosage for a given study. Finally, our focus is ecological, so we included studies in natural ecosystems, greenhouses, or forestry studies but excluded cropland studies.

Because fertilization methodology varied considerably, we express the response variables as ratios that describe the nutrient

concentration of plants after fertilization divided by the nutrient concentration of unfertilized plants. Utilizing ratios enabled comparisons to be made across a wide range of relevant studies. The ln-transformed response ratio (RR) was calculated for all foliar N and P data using the following equations:

$$RR_N = \ln(\text{foliar [N] in fertilized treatment (either } +N, +P, \text{ or } +NP)/\text{foliar [N] in unfertilized treatment})$$

$$RR_P = \ln(\text{foliar [P] in fertilized treatment (either } +N, +P, \text{ or } +NP)/\text{foliar [P] in unfertilized treatment})$$

A summary of relevant information from each publication was entered into an Excel sheet. Foliar N and P concentrations of the control and treatments were entered either on a mass (mg/g) or area (mg/m^2) basis, yet the sample size of values entered by area was too small to analyze. Bytescout Graph Digitizer Scout 1.2.4 was used to identify foliar N and P concentrations when presented in graphical form within the publications.

Appendices A, B in Supplementary Material provides the variables by which the ln of foliar N and foliar P were analyzed. Ecosystem type was determined based on what was described by authors in their site descriptions, or if that was unavailable we used prior knowledge based on latitude and geography to assign an ecosystem class. Forest was considered tropical if it fell in between 23.5°N and S and Arctic Tundra was designated if the site was at ≥66°N; dry forest was defined as <1000 mm mean annual precipitation (MAP) and wet forest as >2500 mm MAP. Other descriptors such as montane and alpine were determined based on topographic position. In addition to ecosystem type we analyzed abiotic factors, temperature and precipitation, as well as organism categorizations, family and life form. Lastly, we only included field fertilization studies but noted the neighborhood in which plants were fertilized. Fertilization could have occurred on individual plants, or on multi-individual monocultures (subject to intraspecific competition) or on mixtures (interspecific competition on a plot level).

One common feature of data used for meta-analyses is that studies differ in their sampling effort and taking not only the mean but also sample sizes and variance into consideration has been recommended (Gurevitch and Hedges, 2001). We did this using MetaWin v. 2 (Rosenberg et al., 2000). We first calculated effect sizes (i.e., response ratios) with corresponding variances and then determined significance based on the $Q_{between}$ term. We used a random effects model which is considered more appropriate for ecological studies (Adams et al., 1997). The model consisted of 5000 resampling tests. Data are presented as means and 95% confidence intervals. Metawin analysis provides a *P*-value for each test conducted, yet mean comparisons output is not provided. Therefore, we inferred significant differences between the categories analyzed based on non-overlapping 95% confidence intervals. We evaluated the problem of publication bias (not publishing negative results as frequently) with the Rosenthal's fail-safe test. Using an alpha value of 0.05 the Rosenthal test tells you the number of values that would need to be added to the analysis to change the significance of the results (Rosenthal, 1979). We also used *t*-tests to evaluate whether means of the response ratios were significantly different from zero.

Control and fertilized N:P ratios were calculated for the entire dataset as well as across ecosystems, families, and life forms. Generally speaking, sample sizes were smaller for these analyses in comparison to the Metawin approach because the dataset used only studies that included both foliar N and P responses to fertilization (Appendices A, B in Supplementary Material). The percent change in N:P following fertilization was calculated as

$$\Delta \text{N:P} = (100 * (\frac{\text{N:P fertilized} - \text{N:P control}}{\text{N:P control}})).$$

RESULTS

Overall Patterns

Across all studies, fertilization with a specific nutrient elicited an increase in foliar concentration of that nutrient. A positive N response occurred after N or NP fertilization (e.g., RR_N significantly > 0), and similarly, a positive P response (RR_P significantly > 0) occurred after fertilization with P or NP (**Table 1**; **Figure 1A**). We also investigated whether the magnitude of each response ratio differed from one another due to the three different types of fertilizations. We found $RR_N > RR_P$ with the addition of N and $RR_P > RR_N$ with the addition of P, but these response ratios were not different from one another when both nutrients were added together (**Table 2**).

TABLE 1 | Summary statistics, including *P*-value, and Rosenthal's fail safe test value, resulting from MetaWin analyses conducted for RR_N and RR_P response ratios with comparisons between nitrogen (N), phosphorus (P), and nitrogen and phosphorus (NP) fertilizations.

Response ratio	Comparison of fertilizations	*P*-value	Rosenthal's	Fertil-ization Type	*n*	Mean	95% CI	*t*-value	*P*-value
RR_N	N vs. P vs. NP	**0.0004**	130862.2	N	420	0.1792	0.0376	16.72	**<0.0001**
				P	65	0.0305	0.0737	1.17	0.246
				NP	253	0.2182	0.0542	15.78	**<0.0001**
RR_P	N vs. P vs. NP	**0.0002**	9601.2	N	125	−0.058	0.0733	−2.98	**0.004**
				P	60	0.2057	0.0974	7.03	**<0.0001**
				NP	147	0.2142	0.0732	6.49	**<0.0001**

Summary statistics resulting from t-test analyses conducted on RR_N and RR_P response ratios when fertilized with N, P, and NP. Summary statistics include sample size (n), mean, 95% confidence interval (CI), t-value and p-value (indicating when mean is significantly different from zero). P-values that are in bold are significant at 0.05 level or less.

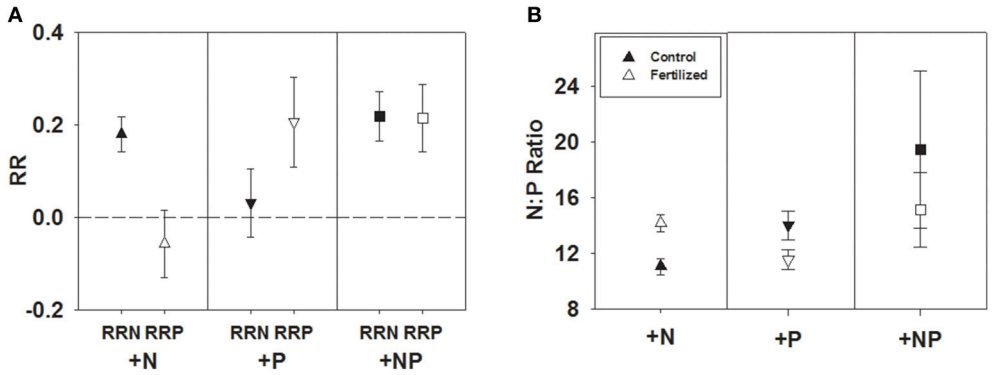

FIGURE 1 | Symbols illustrate mean ± 95 CI differences between RR_N and RR_P response ratios when fertilized with +N, +P, and +NP (A), or N:P, mean ± 95 CI, differences between control and fertilized (+N, +P, +NP) treatments (B).

TABLE 2 | Summary statistics, including *P*-value, and Rosenthal's fail safe test value, resulting from MetaWin analyses conducted for comparisons between RR_N and RR_P response ratios when fertilized with nitrogen (N), phosphorus (P), and nitrogen and phosphorus (NP).

Fertilization	Comparison of responses	*P*-value	Rosenthal's
N	RR_N vs. RR_P	**0.0002**	45887.6
P	RR_N vs. RR_P	**0.0002**	1985.7
NP	RR_N vs. RR_P	0.9592	36056.4

P-values that are in bold are significant at 0.05 level or less.

An alternative way of examining the data is to compare N:P ratios before and after fertilization (+N, +P and +NP). We found N:P ratios to increase significantly after N fertilization [$t_{(79)} = 7.94$, $P < 0.0001$] and decrease significantly after P fertilization [$t_{(49)} = -3.81$, $P < 0.0004$], while no difference in N:P ratios resulted from NP fertilization [$t_{(127)} = -1.38$, $P = 0.1705$; **Figure 1B**].

Climate, Ecosystems, and Competitive Environment

Surprisingly, the two climate variables, temperature and precipitation, were not good predictors of RR_N or RR_P. In some cases there were significant effects, yet the R^2 values were too low to suggest strong relationships (Appendices A and B in the Supplementary Material). Thus, we are unable to draw conclusions of N or P limitation based on precipitation or temperature.

$RR_N > 0$ was seen in 12 of 14 ecosystems fertilized with N and 8 of 15 ecosystems fertilized with NP. Ecosystem responses differed significantly with non-forested wetland showing the largest response and temperate coniferous forest showing the smallest response to both fertilization treatments (**Figures 2A,C**; **Table 3**). N fertilization resulted in 2 of 13 ecosystems (non-forested wetland and temperate coniferous forest) showing a negative RR_P response, indicating a nutrient interaction effect, with no difference between the ecosystems (**Figures 2B,D**; **Table 3**).

$RR_P > 0$ was seen in 5 of 10 ecosystems fertilized with P and 9 of 13 ecosystems fertilized with NP. Ecosystem responses differed significantly with temperate grassland and tropical montane wet forest showing the largest response for the P and NP fertilization treatments, respectively, while temperate deciduous forest showed the smallest response for both treatments (**Figures 2E,F**; **Table 3**).

Competitive environment influenced the foliar nutrient response. Plants fertilized as individuals had a larger RR_N after N or NP fertilization response than those planted in interspecific or intraspecific combinations (**Figures 3A–C**; **Table 3**). For RR_P plants in intraspecific combinations after P fertilization had the largest responses (**Figures 3D,E**; **Table 3**). There was no significant difference in RR_P between the competitive environments when fertilization with NP was conducted (**Figure 3F**; **Table 3**).

Family and Life Forms

RR_N increases were seen in 14 of 33 families fertilized with N, one of 12 fertilized with P, and four of 26 fertilized with NP. Significant differences in RR_N were found between the families with Ericaceae, Cyperaceae, and Ginkgoaceae showing the greatest responses to N fertilization (**Figure 4A**; **Table 3**). Poaceae was the only family that resulted in a positive RR_N response when fertilized with P (**Figure 4B**; **Table 3**), indicating a nutrient interaction for this family. Significant differences of RR_N were not found among the four families (i.e., Ericaceae, Poaceae, Myrtaceae, and Pinaceae) that showed positive responses to NP fertilization (**Figure 4C**; **Table 3**).

A positive RR_P response to P and NP fertilization was found for three of 12 families fertilized with P and six of 22 families fertilized by NP. RR_P response of Ericaceae was found to be greater after P fertilization than Fabaceae, while the response of Cyperaceae, Ericaceae, and Myrtaceae were found to be greater than Fabaceae after NP fertilization (**Figures 4E,F**; **Table 3**). An interaction effect was evidenced by negative RR_P responses to N fertilization for 3 out of 18 families, suggesting an interaction effect. Across these three families Ericaceae had a significantly greater RR_P response than Pinaceae (**Figure 4D**; **Table 3**).

All life forms except ferns demonstrated positive responses to N fertilization, with shrubs showing the greatest RR_N response (**Figures 5A,C**; **Table 3**). An interaction effect was

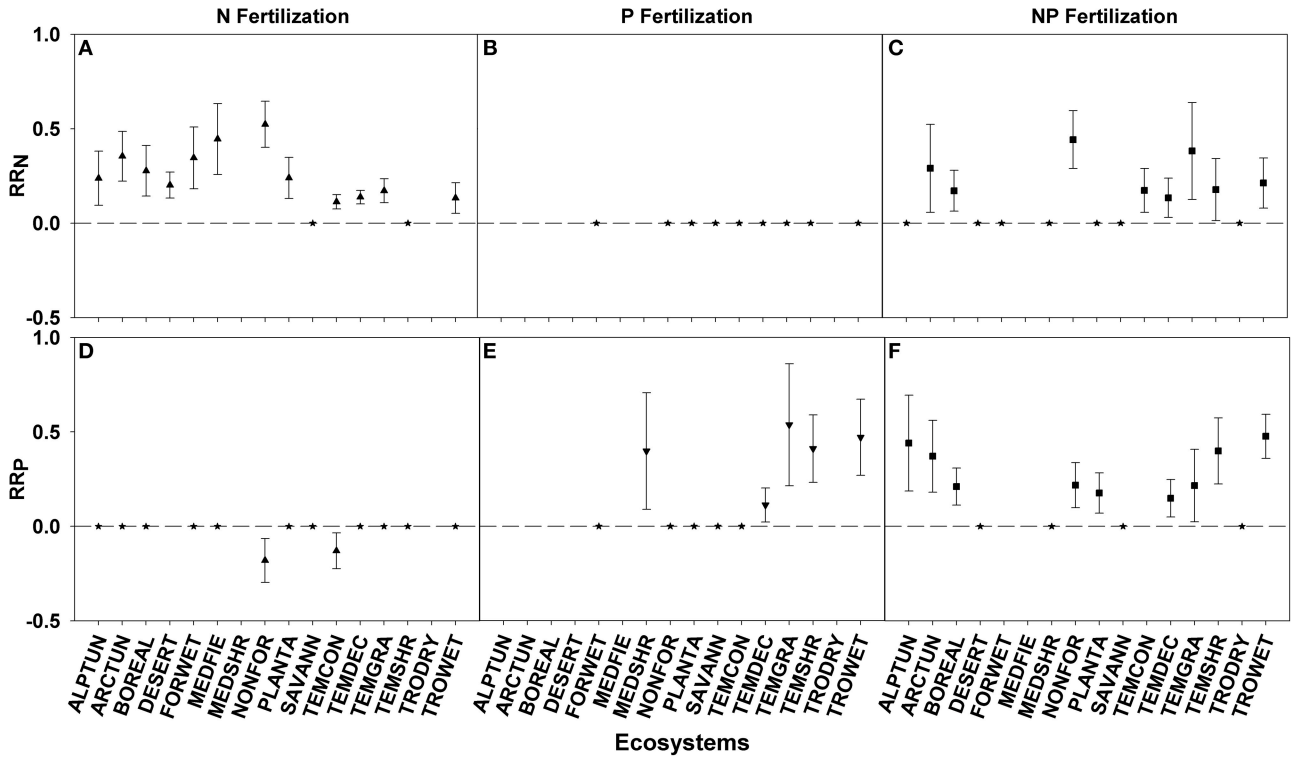

FIGURE 2 | Symbols illustrate RR_N, means ± 95 confidence interval (CI), across ecosystems with nitrogen (N), phosphorus (P), and nitrogen and phosphorus (NP) fertilizations (A–C respectively), or RR_P, means ± 95 confidence interval (CI), across ecosystems with nitrogen (N), phosphorus (P), and nitrogen and phosphorus (NP) fertilizations (D–F respectively). Ecosystems abbreviations used were: ALPTUN, (Alpine Tundra); ARCTUN, (Arctic Tundra); BOREAL, (Boreal Forest); DESERT, (Desert); FORWET, (Forested Wetland); MEDFIE, (Mediterranean Field); MEDSHR, (Mediterranean Shrubland); NONFOR, (Non-forested Wetland); PLANTA, (Plantation); SAVANN, (Savanna); TEMCON, (Temperate Coniferous); TEMDEC, (Temperate Deciduous); TEMGRA, (Temperate Grassland); TEMSHR, (Temperate Shrubland); TRODRY, (Tropical Dry Forest); and TROWET, (Tropical Wet Forest). Asterisks represent RR values that are not significantly different from zero.

found for graminoids which showed a positive RR_N response to P fertilization (**Figure 5B**; **Table 3**). Positive fertilization responses were found for shrubs and trees when fertilized with P and for all life forms except ferns when fertilized with NP (**Figures 5E,F**; **Table 3**). Shrubs had the greatest RR_P response to P fertilization. Yet, no significant differences in RR_P response were found between life forms fertilized with NP. An interaction effect was noted for forbs, shrubs, and trees because of the negative RR_P response to N fertilization (**Figure 5D**; **Table 3**).

Ratios of N:P as an Alternative Indicator

N:P ratios, a common tool used to infer nutrient limitation, were compared across ecosystems, families, and life forms in order to assess whether we can infer N or P limitation based on shifts in N:P ratio following fertilization (**Figures 6**, **7**, see Appendix C in Supplementary Material for sample sizes). For instance, a significantly greater N:P ratio when fertilized with N or NP compared to the control suggests N limitation. Similarly, a smaller N:P ratio when fertilized with P or NP compared to the control could indicate P-limitation.

Using these criteria, we found suggestions of N-limitation for five of the 13 ecosystems, five of the 15 families, and two of the five life forms fertilized with N. Similarly, we found suggestions of P-limitation for four of the 10 ecosystems, four of the 10 families, and one of the four life forms fertilized with P. After NP fertilization we found that the majority of changes across ecosystems, families, and life forms were driven by P concentrations, due to the significant decreases rather than increases in N:P ratios (**Figures 6**, **7**).

Publication Bias and Rosenthal's Value

Rosenthal's fail-safe test provides the number of values that would need to be added to the analysis to change the significance of the results, and therefore serves as a measure of publication bias. Large Rosenthal values provided for the entire dataset analyses indicate low probability of publication bias; all of our values (see **Tables 1, 2**) were larger than the suggested cutoff of 5N +10, where N = number of cases (Rosenberg et al., 2000). It should be noted though that Rosenthal's values were much lower for RR_N responses to P fertilizations and RR_P responses to N fertilizations (**Table 3**), a likely publication bias because very few P fertilization studies measure N concentrations (see Appendices A, B in Supplementary Material for sample sizes). See Appendix D in

TABLE 3 | Summary statistics, including *P*-value, and Rosenthal's fail safe test value, resulting from MetaWin analyses conducted for RR_N and RR_P response ratios when fertilized with nitrogen (N), phosphorus (P), and nitrogen and phosphorus (NP) across categories: ecosystem type, competitive environment, family, and life form.

Response Ratio	Category	N Fertilization		P Fertilization		NP Fertilization	
		P-value	Rosenthal's	*P*-value	Rosenthal's	*P*-value	Rosenthal's
RR_N	Ecosystem Type	**0.0114**	50703.2	0.7023	0	**0.0002**	11642.2
	Competitive Environment	**0.0048**	54159.6	0.6309	0	0.015	12367
	Family	**0.0304**	55652.4	0.3487	0	0.1318	9603.4
	Life Form	**0.0004**	55008.2	0.1254	0	0.0172	14696.1
RR_P	Ecosystem Type	0.1098	303.6	**0.0008**	1987.7	**0.0004**	7489.3
	Competitive Environment	0.058	282.1	**0.0062**	1605	0.1268	5894.9
	Family	**0.0418**	179.4	**0.0052**	1515	0.0002	9133.1
	Life Form	0.1458	233	**0.0152**	1602.1	0.1608	6450.3

P-values that are in bold are significant at 0.05 level or less.

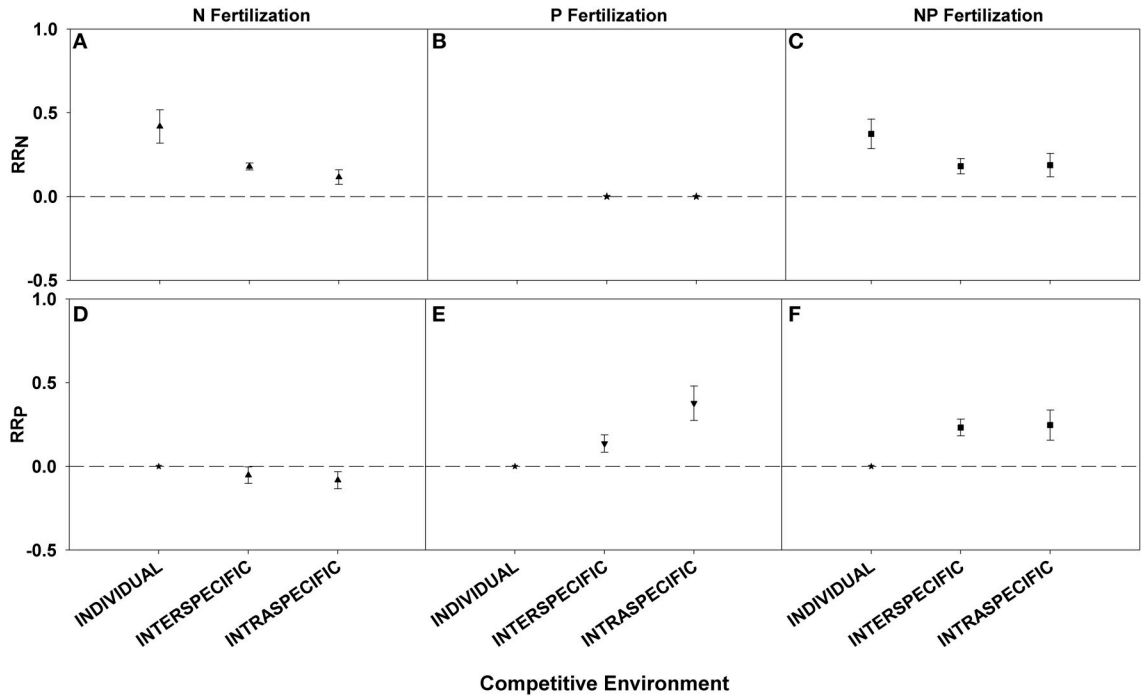

FIGURE 3 | Symbols illustrate RR_N, means ± 95 confidence interval (CI), across competitive environments: individual, interspecific, and intraspecific, with nitrogen (N), phosphorus (P), and nitrogen and phosphorus (NP) fertilizations (A–C respectively), or RR_P, means ± 95 confidence interval (CI), across competitive environments: individual, interspecific, and intraspecific, with nitrogen (N), phosphorus (P), and nitrogen and phosphorus (NP) fertilizations (D–F respectively). Asterisks represent RR values that are not significantly different from zero.

Supplementary Material for a list of studies used in the meta-analysis.

DISCUSSION

Detecting Nutrient Limitation and the Stoichiometry of N and P

Regardless of whether response ratios (RR_N or RR_P) or N:P values are considered (**Figures 1A–D**), there is strong evidence for N and P limitation in natural communities. Our results mirror those of Elser et al. (2007), who conducted similar types of analyses on nutrient enrichment across terrestrial, freshwater, and marine ecosystems, but who used biomass as a response variable. The consistency among the response variables is remarkable, given that foliar nutrient concentrations vary for many reasons not analyzed in this study (e.g., with laboratory methods, age of leaves, canopy position, seasonality, study duration, fertilization dosage, and type). Not only are foliar

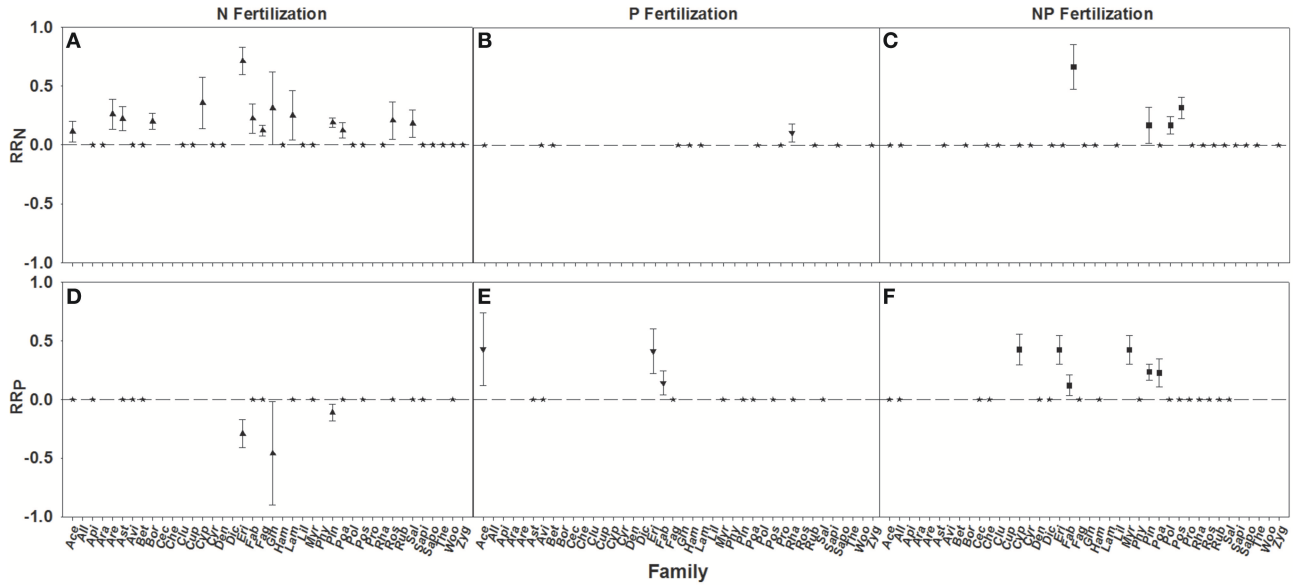

FIGURE 4 | Symbols illustrate RR_N, means ± 95 confidence interval (CI), across familes, with nitrogen (N), phosphorus (P), and nitrogen and phosphorus (NP) fertilizations (A–C respectively), or RR_P, means ± 95 confidence interval (CI), across families, with nitrogen (N), phosphorus (P), and nitrogen and phosphorus (NP) fertilizations (D–F respectively). Family abbreviations used were: Ace, (Aceraceae); All, (Alliaceae); Api, (Apiaceae); Ara, (Araceae); Are, (Arecaceae); Ast, (Asteraceae); Avi, (Avicenniaceae); Bet, (Betulaceae); Bor, (Boraginaceae); Cec, (Cecropiaceae); Che, (Chenopodiaceae); Clu, (Clusiaceae); Cup, (Cupressaceae); Cyp, (Cyperaceae); Cyr, (Cyrillaceae); Den, (Dennstaedtiaceae); Dic, (Dicksoniaceae); Eri, (Ericaceae); FAB, (Fabaceae); Fag, (Fagaceae); Gin, (Ginkgoaceae); Ham, (Hamamelidaceae); Lam, (Lamiaceae); Lil, (Liliaceae); Myr, (Myrtaceae); Phy, (Phytolaccaceae); Pin, (Pinaceae); Poa, (Poaceae); Pol, (Polygonaceae); Pos, (Posidoniaceae); Pro, (Proteaceae); Rha, (Rhamnaceae); ROS, (Rosaceae); RUB, (Rubiaceae); SAL, (Salicaceae); Sapi, (Sapindaceae); Sapo, (Sapotaceae); The, (Theaceae); Woo, (Woodsiaceae); and Zyg, (Zygophyllaceae). Asterisks represent RR values that are not significantly different from zero.

nutrients logistically simpler than measuring plant growth rates, but in the absence of nutrient supplementation experiments, foliar nutrient analysis will provide a relative assessment of nutrient status (Tanner et al., 1998).

Contrary to our predictions, but in agreement with Elser et al. (2007), single nutrient additions of N and P when compiled across many studies were approximately equivalent in response (i.e., RR_N after +N addition and RR_P after +P addition, **Figure 1**). There are many examples in individual studies, within a single ecosystem or biome, that suggest that P concentrations are inherently greater in magnitude and more variable than foliar N concentrations (Güsewell and Koerselman, 2002; Campo and Dirzo, 2003; Güsewell, 2004, 2005; Townsend et al., 2007; Ågren, 2008; Ostertag, 2010; Mayor et al., 2014). In the global aggregate however, this meta-analysis supports the tight stoichiometric coupling of N and P across ecosystems, taxa, and life forms.

The fact that N and P varied about the same order of magnitude in response to fertilization also suggests that N:P can be a useful index of nutrient limitation. We saw a general pattern that with N fertilization N:P values increased (also seen in a meta-analysis by Sardans et al., 2012), and with P fertilization N:P decreased. Indeed, leaf N:P values have been widely adopted in the literature as indications of N or P limitation (Güsewell and Koerselman, 2002; Townsend et al., 2007; Ågren, 2008). Initial work identified values <14 as indicative of N-limitation and >16 as indicative of P limitation (Koerselman and Meuleman, 1996). In the ecosystems surveyed here in which RR_N indicated N limitation, N:P values ranged from 3.8 to 19.3 (mean = 10.5) and for those in which RR_P indicated P limitation, N:P values ranged from 9.2 to 17.7 (mean = 13.0). Given the large range, we agree with others that have pointed out that the ratios provide useful information when used in a comparative context, but there are unlikely to be universal cutoff values that determine the type of nutrient limitation (Tessier and Raynal, 2003; Drenovsky and Richards, 2004; McGroddy et al., 2004; Soudzilovskaia et al., 2005; Townsend et al., 2007; He et al., 2008). Ratios of N:P vary with more than just soil nutrient availability; for instance, values can be influenced by species and phylogeny (Townsend et al., 2007), seasonality (Townsend et al., 2007; Rivas-Ubach et al., 2012), environmental stress (Rivas-Ubach et al., 2012), and tissue type and age (Schreeg et al., 2014). Thus, we strongly suggest that determining a cut-off value to indicate N or P limitation should be a site-specific endeavor.

Under +NP fertilization, examination of foliar nutrients did not yield a synergistic nutrient response, unlike meta-analyses that focused on biomass production (Elser et al., 2007; Harpole et al., 2011). New growth causing a dilution of nutrient concentrations is the most logical explanation for our results (Jarrell and Beverly, 1981). In addition, the timing of sample collection relative to fertilization events may be important, as foliar nutrient changes may be shorter-lived or occur before growth responses, making them difficult to detect. For example, in the Alaskan tundra, Shaver and Chapin (1995) demonstrated that growth responses could occur a year after fertilization; in spruce and pine stands a single fertilization can affect conifer growth for 7–10 years (Pettersson and Högbom, 2004). The

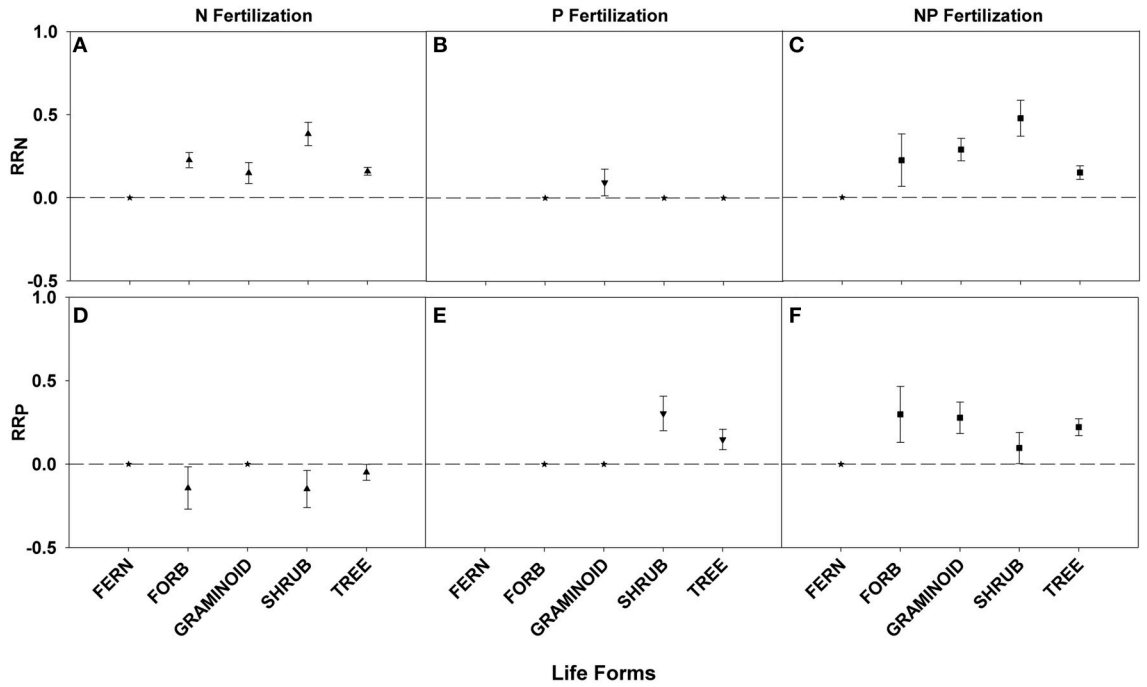

FIGURE 5 | Symbols illustrate RR_N, means ± 95 confidence interval (CI), across life forms: fern, forb, graminoid, shrub, and tree, with nitrogen (N), phosphorus (P), and nitrogen and phosphorus (NP) fertilizations (A–C respectively). Symbols illustrate RR_P, means ± 95 confidence interval (CI), across life forms: fern, forb, graminoid, shrub, and tree, with nitrogen (N), phosphorus (P), and nitrogen and phosphorus (NP) fertilizations (**D–F** respectively). Asterisks represent RR values that are not significantly different from zero.

timing of the foliar nutrient increases after fertilization in relation to growth responses is generally unknown. More mechanistic studies are required to determine the level of decoupling of foliar nutrient concentration and growth responses under a wide range of environmental conditions.

This meta-analysis contains hints of multiple elemental limitations, which deserves much more intense study through experimentation, given that it may be common in terrestrial ecosystems (Vitousek et al., 2010; Harpole et al., 2011; Wright et al., 2011). A consistent result across the entire data set was the interaction between N and P (**Figure 1**); fertilizing with N decreases RR_P by about 130% and fertilizing with P decreases RR_N by about 80%. Similar patterns were noted across ecosystems, families, and life forms, particularly for the decrease in RR_P with +N. Why do we see this asymmetry?

In the case of N fertilization affecting foliar P levels, several studies demonstrate that N fertilization increases phosphatase enzyme activity within ecosystems (Treseder and Vitousek, 2001; Wang et al., 2007), for both plant and soil phosphatases (Marklein and Houlton, 2012). The additional N plants receive via fertilization may therefore be allocated to phosphatase activity, enhancing P uptake, which in theory should lead to increased foliar P concentrations. This result was seen in a regional study in the northeastern US, where higher foliar P concentrations were noted in areas with higher N deposition rates (Crowley et al., 2012). Allocation to mycorrhizae may be an alternative or synergistic mechanism; for example, nitrogen-fixing trees in lowland wet tropical forests in Costa Rica had greater phosphatase enzyme activity and greater arbuscular mycorrhizal colonization than non-fixers (Nasto et al., 2014). However, a contrasting hypothesis better fits the data from this meta-analysis: that additional N inputs push a system closer to N saturation, shifting the balance toward P limitation, perhaps because the accompanying soil acidification slows P mineralization (Harrison, 1982), or because there is less effective P uptake via negative effects of fertilization on root biomass, length, and/or mycorrhizal activity and diversity (Ostertag, 2001; Treseder, 2004; Porras-Alfaro et al., 2007). It is also important to note that the additional N does not quantitatively add new P molecules to the ecosystem, but only changes the cycling rates (Vitousek et al., 2010).

In the case of P fertilization affecting foliar N levels, Vitousek et al. (2010) suggested that fertilizing with P can actually quantitatively increase the amount of N in an ecosystem, due to the stimulation of N-fixation by N-fixing plants and microbes. Evidence for this hypothesis comes from greater N-fixation rates in P-fertilized plots in a tallgrass prairie (Reed et al., 2007); in theory, N-fixing plants should have higher foliar N concentrations. While this mechanism is plausible, it seems unlikely to explain our results. First, the response is in the wrong direction because in our meta-analysis, there is a decline in RR_N relative to RR_P. Second, many of the studies analyzed in this meta-analysis did not feature N-fixing plant species, although the

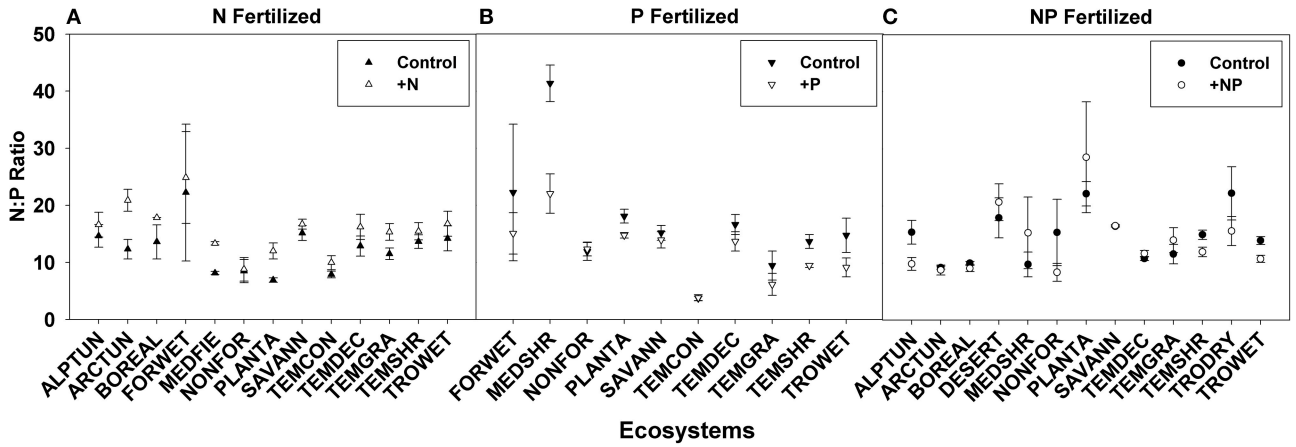

FIGURE 6 | Symbols illustrate N:P ratio, mean ± 95 CI, differences between control and fertilized (+N, +P, +NP) treatments (A–C respectively) across ecosystems. Ecosystem abbreviations used were: ALPTUN, (Alpine Tundra); ARCTUN, (Arctic Tundra); BOREAL, (Boreal Forest); DESERT, (Desert); FORWET, (Forested Wetland); MEDFIE, (Mediterranean Field); MEDSHR, (Mediterranean Shrubland); NONFOR, (Non-forested Wetland); PLANTA, (Plantation); SAVANN, (Savanna); TEMCON, (Temperate Coniferous); TEMDEC, (Temperate Deciduous); TEMGRA, (Temperate Grassland); TEMSHR, (Temperate Shrubland); TRODRY, (Tropical Dry Forest); TROWET, (Tropical Wet Forest).

possibility of free-living N-fixers in the soil being stimulated by the external P inputs cannot be ruled out (Reed et al., 2011). What seems plausible to explain the meta-analysis results is that P fertilization is shifting ecosystems toward N limitation; Vitousek et al. (2010) outlines this possibility in P-fertilized ecosystems or those where atmospheric dust containing P is transferred long distances.

Trends in RR_N and RR_P across Climatic Gradients and Ecosystems

Surprisingly, there were not strong patterns in RR_N and RR_P across global climate gradients of mean annual temperature and precipitation. We expected to find increasing RR_N and RR_P with increasing temperatures based on studies that have shown foliar N and P to decrease with increasing temperatures (McGroddy et al., 2004; Reich and Oleksyn, 2004; Han et al., 2005; Sardans et al., 2011). Furthermore, we expected to find increasing response ratios, especially RR_P, with increasing levels of precipitation based on a global meta-analysis that found P availability in soil to decrease with increasing precipitation (Ordoñez et al., 2009). At a smaller scale, foliar P was correlated with mean annual precipitation gradient (350–1400 mm) across >3500 plots representing the Mediterranean ecosystems of NE Spain (Sardans et al., 2011). We may not have found a strong trend across these climatic variables due to confounding factors, such as community composition, substrate, and altitude. For example, similar to our results, Alvarez-Clare and Mack (2011) found in wet tropical forests that mean annual precipitation was not correlated with foliar N or P. The abundance of legumes was suggested as a confounding factor because the legume abundance varied across their rainfall gradient (3500–5500 mm). In addition, this meta-analysis may differ from temperature and rainfall gradient studies for two important reasons: (1) we assessed changes in foliar concentrations following fertilization rather than solely trends in foliar quality across climatic gradients, and (2) our study included a wider range of mean annual temperature and precipitation values than other literature (−13.7–32.0°C and 102–7800 mm).

In contrast, comparisons of foliar nutrient responses across ecosystem categories suggest nutrient limitations. Two previous meta-analyses focusing on N limitation are in agreement with our results, although both of these focused on ANPP and not foliar nutrient concentrations. LeBauer and Treseder (2008) showed that across all studies there was no correlation between ANPP response ratio and latitude, MAP, or MAT, but they did find that N limitation was evident in temperate forest, tropical forest, temperate grassland, tropical grassland, wetlands, and tundra, which agree fully with our results (Appendix A in Supplementary Material). In addition, Yahdjian et al. (2011) focused more intensively on arid-subhumid ecosystems (MAP/PET ratio from 0.05 to 0.75), which they argued have been under-sampled in the two other main meta-analyses (Elser et al., 2007; LeBauer and Treseder, 2008). They found that ANPP after N fertilization increased by 50% across all studies; we found that deserts responded positively to N fertilization. It is therefore encouraging that all studies converge on the same conclusions despite using different response variables. It is also encouraging that their analyses found that the effect of N-application rate, and duration of time from fertilization to field measurement (LeBauer and Treseder, 2008), and the form of fertilizer applied (LeBauer and Treseder, 2008; Yahdjian et al., 2011) did not significantly influence the growth response. These results provide reassurance that fertilizer studies can be compared, despite differences in methodology.

Because N fertilization experiments greatly outnumber P fertilization trials, it is difficult to discern the absolute importance of N vs. P vs. co-limitation across ecosystems. In addition, as pointed out by Sullivan et al. (2014), fertilization experiments

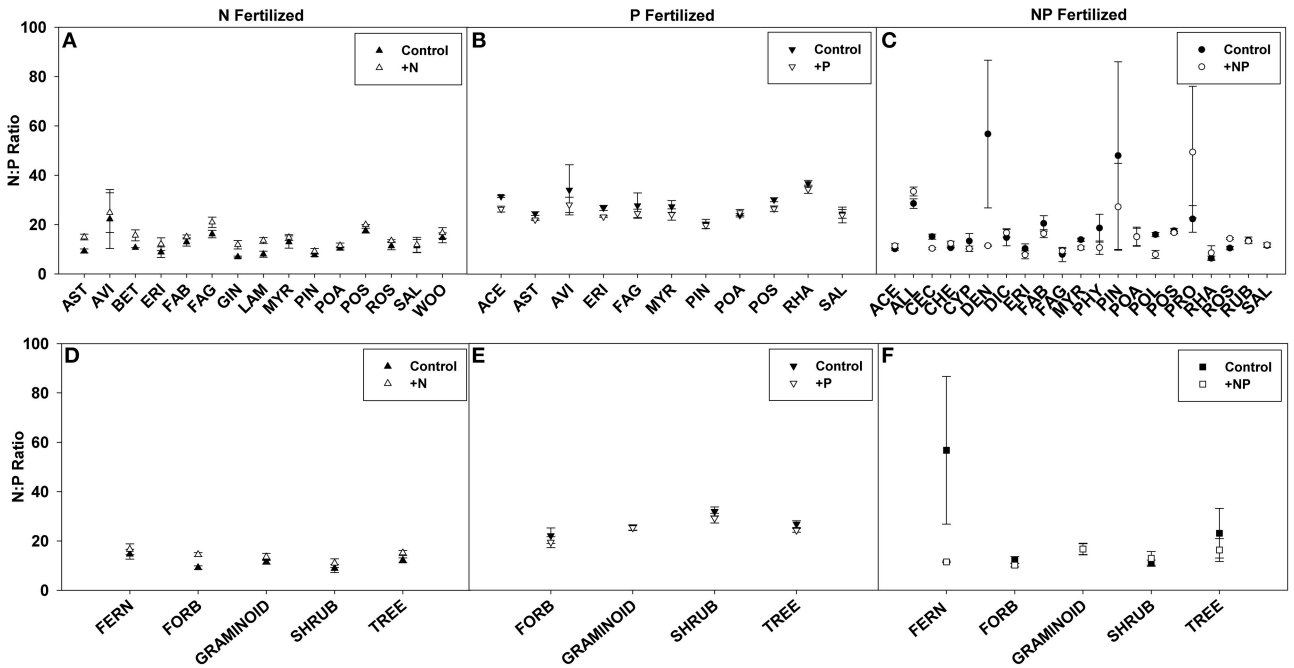

FIGURE 7 | Symbols illustrate N:P ratio, mean ± 95 CI, differences between control and fertilized (+N, +P, +NP) treatments (A–C respectively) across families. Family abbreviations used were: ACE, (Aceraceae); ALL, (Alliaceae); AST, (Asteraceae); AVI, (Avicenniaceae); BET, (Betulaceae); CEC, (Cecropiaceae); CHE, (Chenopodiaceae); CYP, (Cyperaceae); DEN, (Dennstaedtiaceae); DIC, (Dicksoniaceae); ERI, (Ericaceae); FAB, (Fabaceae); FAG, (Fagaceae); GIN, (Ginkgoaceae); LAM, (Lamiaceae); MYR, (Myrtaceae); PHY, (Phytolaccaceae); PIN, (Pinaceae); POA, (Poaceae); POL, (Polygonaceae); POS, (Posidoniaceae); PRO, (Proteaceae); RHA, (Rhamnaceae); ROS, (Rosaceae); RUB, (Rubiaceae); SAL, (Salicaceae); and WOO, (Woodsiaceae). Symbols illustrate N:P ratio, mean ± 95 CI, differences between control and fertilized (+N, +P, +NP) treatments (**D–F** respectively) across life forms: fern, forb, graminoid, shrub, and tree.

are more common in ecosystems with short-statured plants, such as grassland, shrubland, and tundra. While our data set does have a considerable number of forest studies (see Appendices A, B in Supplementary Material for sample sizes), the sample sizes are quite unequal among ecosystems. Despite these caveats, it is striking that our results did not follow "conventional wisdom" that temperate ecosystems are N-limited and tropical ones are P-limited, a conclusion based on soil age, weathering processes, and litterfall patterns (Walker and Syers, 1976; Vitousek, 1984; McGroddy et al., 2004). If we assume that foliar response can detect nutrient limitation, then alpine tundra, Arctic tundra, boreal forests, non-forested wetlands, plantations, temperature coniferous forest, temperate deciduous forest, temperate grasslands, and tropical wet forest were all both N-limited and NP co-limited. Furthermore, temperate deciduous forest, temperate grasslands, temperature shrublands and tropical wet forest showed patterns of being both P-limited and NP co-limited. These results agree with other recent studies that have found co-limitation to occur in temperate (Niinemets and Kalevi, 2005; Vadeboncoeur, 2010) and tropical (Wullaert et al., 2010) ecosystems. P-limitation or co-limitation in temperate systems might occur because: (1) until recently P limitations have not been investigated as rigorously as N limitations in the temperate systems (Vadeboncoeur, 2010), and (2) increased N deposition and its physiological consequences are shifting the nature and extent of nutrient limitations (Von Oheimb et al., 2010). Similarly, some tropical forests have been found to have N-limitation due to substrate age and type that does not fit the norm for tropical systems (Domingues et al., 2010). This study and two others (Elser et al., 2007; LeBauer and Treseder, 2008) all demonstrate that there does not appear to be a consistent latitudinal trend in nutrient limitation. We argue that a broader mindset needs to be employed when considering nutrient limitation, taking into account that many factors beyond soil age and climate. Some of these factors are: soil alkalinity (Drenovsky and Richards, 2005), fire (Sardans et al., 2006; Stock and Verboom, 2012), flooding (Day, 1987; Feller et al., 2007), size class (Alvarez-Clare et al., 2013), species and phylogeny (Alvarez-Clare et al., 2013), and anthropogenic disturbance (Gariola et al., 2009).

Influences of Taxonomy and Attributes of Experimental Design on RR_N and RR_P

It is important to note that this study cannot make definitive conclusions about the relative importance of N and P limitation for these families, because of the paucity of fully reciprocal fertilization experiments. In addition, key families such as the Proteaceae, known to thrive under low nutrient conditions (Witkowski, 1990; Lambers and Shane, 2007; Stock and Verboom, 2012) and to have adaptations for efficient P use (de Campos et al., 2013), could not be included due to low sample sizes (i.e., not enough fertilization experiments). However, some interesting phylogenetic patterns were observed. Families that demonstrated nutrient limitation were often those that are

dominants in nutrient-poor ecosystems. For example, Ericaceae is able to thrive on acidic and infertile soils due to special root mycorrizhal associations with fungi that maximize its uptake of nutrients (Lambers et al., 2008), and in this study the family was suggested to be N-, P-, and co-limited. Cyperaceae also has specialized root adaptations for low P soils (Lambers et al., 2008) and is common in many herbaceous communities; it was likely to be N- and co-limited. Two notable ectomycorrhizal families were Pinaceae, abundant in temperate coniferous and boreal forests and Fagaceae, often found on low-nutrient soils associated with temperate forests, Mediterranean shrublands, and tropical montane forests (Manos and Stanford, 2001). Finally, Fabaceae responded to fertilization with P and NP; as many species are nitrogen-fixing, the N added to soils from these species could be influencing the rates of P cycling (Vitousek et al., 2010).

Differences among life forms were also noted. A meta-analysis of foliar nutrient concentrations conducted on 753 Chinese species found N and P concentrations to be higher in herbs than woody plants (Han et al., 2005). The difference was explained by opposite growth form strategies, in which herbs are short-lived and fast-growing thus cycling nutrients at a faster rate than the longer-lived slow-growing woody species. Our meta-analysis suggested co-limitation for herbs, graminoids, shrubs, and trees but the significant differences in responses between life forms are difficult to decipher. The lack of clear patterns across the different life forms is most likely due to interactions with the families and ecosystems in which they exist.

We conducted an analysis across competitive environments in order to assess if this detail of experimental design might influence the magnitude of nutrient response. We found greater RR_N for plants fertilized individually, rather than for plants with other individuals and thus undergoing inter- or intra-specific competition. These results were expected because growing a plant individually removes competition, thus allowing that plant greater access to the nutrients provided. The small pool of P data available for plants fertilized individually made it difficult to draw conclusions on this category, but we would have expected RR_P to follow the same pattern seen in RR_N. There was no difference between combinations of plants for RR_N, yet for RR_P the response was greater when plants were fertilized in intra-specific combinations than inter-specific combinations. However, based on niche partitioning theory we would have expected the opposite result: mixtures of species would have greater nutrient levels because of access to different nutrient pools via mechanisms such as rooting depth (Reynolds et al., 2003; Rennenberg and Schmidt, 2010). Evidence for this complementarity mechanism is readily available in the forestry literature comparing monocultures and polycultures (Oelmann et al., 2010; Richards and Schmidt, 2010). A larger sample size and more equal sampling among categories are required to make definitive conclusions.

Sample Size and Publication Bias

Historically, N has been a nutrient well studied in terrestrial systems. It has not been until recent years that the importance of P limitation for these ecosystems has become documented and that more studies have begun to examine the ecology surrounding P. This is evident in the much smaller sample sizes for RR_P found in our dataset. Unfortunately, this made it impossible to make some cross-comparisons between categories for RR_N and RR_P. In assessing the Rosenthal's values across the different categorical analyses we found the power of our results weakened by poor sample size for RR_N with +P and to a lesser extent RR_P with +N (**Table 3**). Uneven sample sizes are also evident across ecosystems and this has a cascading effect on other categories such as families and life forms. For instance, temperate coniferous and deciduous forests have 3-10-fold greater sample sizes than the other ecosystems, Pinaceae and Fagaceae were very well represented, and contributed to the number of trees being 3-80-fold greater than the other life forms.

CONCLUSIONS

The value of meta-analysis—combining many studies together to discern broad scale patterns—is also its weakness, in that fertilization experiments differ in many ways. Meta-analyses will always have bias (Osenberg et al., 1999). In this case, the ecosystems where ecologists have worked is non-random, publication rates may be lower for negative results, there are fewer studies on P, and differences in laboratory analytical techniques in nutrient measurements might influence N and P values. Yet, despite these known factors our overall results were strongly significant and consistent across categories. Therefore, meta-analysis has proved a valuable tool in examining foliar nutrient responses to fertilization, providing evidence for the following: (1) magnitudes of response to +N and +P are similar for RR_N and RR_P, respectively, (2) parallel patterns exist in RR_N and RR_P to what would be predicted with N:P stoichiometric theory, (3) climate is not a good predictor of RR_N or RR_P, but response ratios do vary in predictable ways across families, ecosystems, life forms, and competitive situations, and (4) fertilization studies show interactions between N and P and evidence of co-limitation in many situations. In order to understand N/P interactions, we put out a methodological plea: studies need to measure both N and P simultaneously because our sample sizes clearly show that most individual experiments that fertilize with one nutrient usually do not measure the other nutrient in plant tissue. While foliar nutrient concentrations are just one tool in the toolbox (sensu Sullivan et al., 2014) to determine nutrient limitation, the fact that our results confirm known ecological patterns about ecosystems offers reassurance that foliar nutrient responses to fertilization are a valuable and simple assessment of nutrient limitation.

AUTHOR CONTRIBUTIONS

ND compiled and analyzed the data, made figures and tables, RO and ND wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/feart.2016.00023

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Nutrient addition effects on tropical dry forests: a mini-review from microbial to ecosystem scales

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Humans have more than doubled inputs of reactive nitrogen globally and greatly accelerated the biogeochemical cycles of phosphorus and metals. However, the impacts of increased element mobility on tropical ecosystems remain poorly quantified, particularly for the vast tropical dry forest biome. Tropical dry forests are characterized by marked seasonality, relatively little precipitation, and high heterogeneity in plant functional diversity and soil chemistry. For these reasons, increased nutrient deposition may affect tropical dry forests differently than wet tropical or temperate forests. Here, we review studies that investigated how nutrient availability affects ecosystem and community processes from the microsite to ecosystem scales in tropical dry forests. The effects of N and P addition on ecosystem carbon cycling and plant and microbial dynamics depend on forest successional stage, soil parent material, and rainfall regime. Responses may depend on whether overall productivity is N- vs. P-limited, although data to test this hypothesis are limited. These results highlight the many important gaps in our understanding of tropical dry forest responses to global change. Large-scale experiments are required to resolve these uncertainties.

Keywords: tropical dry forest, nutrient limitation, ecosystem processes, carbon cycling, decomposition, nitrogen deposition, nitrogen cycling

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Introduction

Not only have humans more than doubled the amount of reactive nitrogen (N) entering the global N cycle through industry, energy use, and agriculture (Vitousek, 1994; Galloway et al., 2004), we have also greatly accelerated the biogeochemical cycles of phosphorus (P) and metals (Smil, 2000; Rauch and Pacyna, 2009). While ecosystem processes in the temperate zone are thought to be primarily limited by the availability of N, a wide array of elements including N, P, and sodium (Na) can control tropical forest productivity, decomposition, and tree species distributions (Kaspari et al., 2009; Cleveland et al., 2011; Condit et al., 2013). The high biogeochemical heterogeneity of tropical forests (Townsend et al., 2008) challenges our ability to model ecosystem carbon (C) cycling and forest dynamics under rapidly changing climate and nutrient deposition regimes, and suggests that research from temperate forests may not apply in the tropics.

Ecosystem state factors including climate, parent material, topography, biotic organisms, and weathering affect the absolute and relative availabilities of different elements (Jenny, 1941).

In general, ecosystem processes (e.g., net primary productivity, NPP) are limited by N on geologically young soil substrates, such as recently glaciated soils in the temperate zone or recent lava flows in the tropics (Vitousek, 1999; Reich and Oleksyn, 2004). By contrast, older geologic substrates are depleted of rock-derived elements such as P and cations [e.g., calcium (Ca), potassium (K)]. As a consequence, it is generally thought that tropical forests, which tend to have more highly weathered soils, are primarily limited by P (Walker and Syers, 1976; Chadwick et al., 1999; Cleveland et al., 2011). Indirect evidence tends to support this generalization for tropical wet forests (Cleveland et al., 2011), although stand-level fertilization experiments demonstrate that N, P, and K additions all may influence tropical NPP (Wright et al., 2011; Alvarez-Clare et al., 2013). Additionally, previous land-use practices such as deforestation and burning may lead to N limitation of young secondary forests (Kauffman et al., 1993; Campo and Vázquez-Yanes, 2004; Davidson et al., 2007).

Tropical dry forests (TDFs) comprise more than 40% of the potentially forested area in tropical latitudes (Murphy and Lugo, 1986). In general, these forests have lower annual rainfall compared to moist or wet tropical forests, and experience a 3+ month dry season with little or no rainfall. As a consequence, many dry forest species have unique adaptations to seasonal drought such as deciduous leaf habits and deep roots (Eamus, 1999). Despite their large area, nutrient limitation and biogeochemical cycling in TDFs are relatively understudied (Gei and Powers, 2014). Further, there are reasons to expect that nutrient constraints and responses to anthropogenic nutrient deposition may differ in dry vs. wet tropical forests because of the strong water limitation and seasonally pulsed soil water availability in TDFs (Lambert et al., 1980; Read and Lawrence, 2006).

Here, we review the few studies that investigate how nutrient availability affects ecological processes in TDFs. Because of rapidly increasing rates of N deposition across the tropics (Hietz et al., 2011; Sullivan et al., 2014a), most of the studies included in our review focus on effects of N addition. We develop a conceptual model of how added N affects TDFs; because TDFs may be N- or P-limited, our model considers how responses to N addition may vary between forests with low or high P availability. We compare these hypotheses with what is known about these interactions in TDFs, integrate key lessons from temperate-zone research and draw contrasts with tropical wet forests. Further, when available, we point to studies that measure TDF ecosystem responses to the addition of P or other elements. We conclude by highlighting research gaps and priorities for future research.

Conceptual Model of Nutrients and Ecological Processes in Tropical Dry Forest

Identifying which elements limit ecological processes in TDFs is a high priority for several reasons. First, ecosystem responses to nutrient enrichment and rising atmospheric CO_2 depend upon the identity of the limiting nutrient (Hall and Matson, 1999). For example, N deposition may increase productivity in N-limited forests, but have no effect in a P-limited forest. Second, because rainfall is highly seasonal in TDFs, documenting interactions between water and nutrient availability may be crucial to accurately predict ecosystem responses to anthropogenic change. Understanding the relationship between precipitation pulses and element cycling poses a significant challenge for ecosystem modelers (Manzoni et al., 2014), and therefore responses of tropical wet forests to fertilization may not generalize to more seasonal ecosystems. Last, TDFs typically contain trees with diverse phenological strategies, from evergreen to drought-deciduous trees (Eamus, 1999), and these may respond differently to changing nutrient availability.

Our conceptual model of how increasing N deposition may affect key ecosystem processes in TDFs includes multiple levels of organization (**Figure 1**). This model summarizes hypotheses about how N deposition interacts with underlying nutrient limitation to generate a diversity of ecosystem responses. We emphasize here that different plant functional or phenological groups may vary in their responses to N deposition. Furthermore, TDFs are notable for the high abundances and diversity of legume trees that fix atmospheric nitrogen, and theory suggests legumes will be less affected by added N (Hedin et al., 2009). Because available evidence on TDF responses to water and nutrient availability is so limited, we treat **Figure 1** as a set of hypotheses, and throughout the review we evaluate the extent to which current literature for all biomes, and TDFs in particular, supports our conceptual model (**Table 1**).

Communities: Microbial Composition and Function

Because bacteria and fungi mediate soil element cycling, microbial responses to N and P inputs may drive ecosystem-scale responses to nutrient enrichment. For example, resource stoichiometry influences microbial growth rates (Rousk and Baath, 2007), substrate use efficiency (Sinsabaugh et al., 2013), and the production of extracellular enzymes that degrade soil organic matter (Mooshammer et al., 2012). In turn, these microbial traits and processes affect soil C storage (Cotrufo et al., 2013) and the availability of nutrients to plants. Across the globe, the C:N:P ratio of microbial biomass is fairly well-constrained despite substantial variation in the environmental availability of these elements (Cleveland and Liptzin, 2007). Therefore, background nutrient limitation will determine microbial responses to nutrient enrichment in TDF. The addition of limiting nutrients should augment the size and/or growth efficiency of the microbial biomass (Schimel and Weintraub, 2003; Geisseler and Scow, 2014), which in turn may alter soil C storage (Bradford et al., 2013; Frey et al., 2014).

Limited evidence suggests that microbial biomass tends to be more P-limited in tropical soils (Cleveland et al., 2002, 2003; Waring et al., 2013; Turner and Wright, 2014; Warren et al., 2015). Consistent with this hypothesis, P but not N addition increased microbial biomass in a TDF in southern China (Li et al., 2015a). However, because diffusion and osmotic stress constrain microbial growth under dry conditions (Manzoni et al. 2014), nutrient uptake is strongly mediated by water availability. The handful of studies that have examined microbial nutrient limitation in TDF soils report that microbial responses to labile C

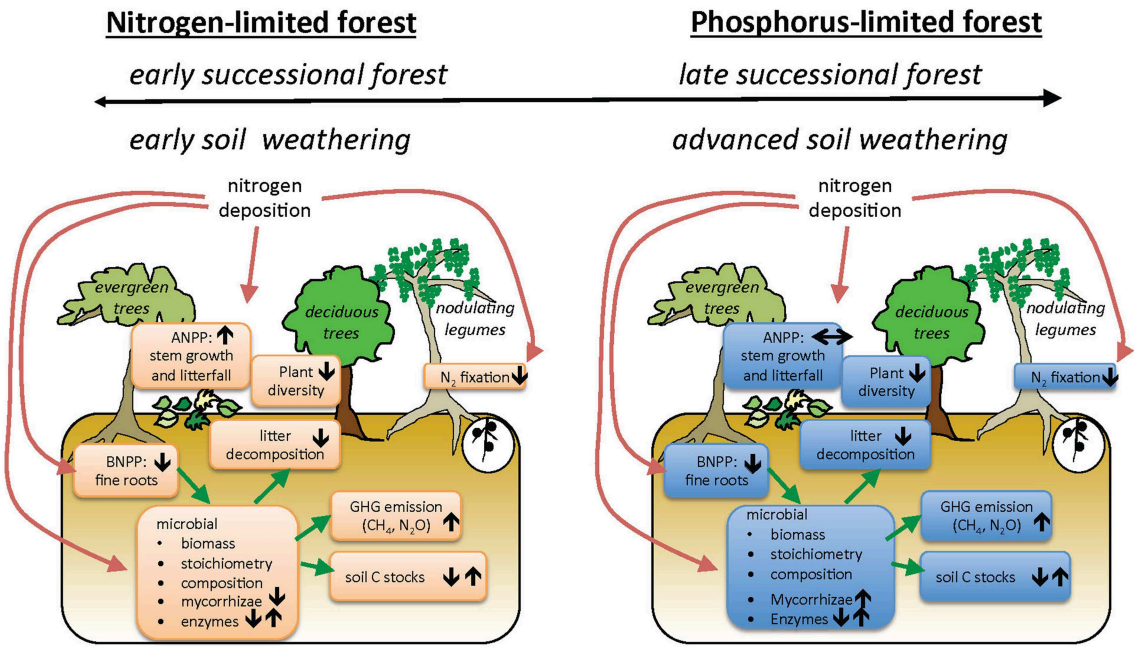

FIGURE 1 | Conceptual model of how nitrogen addition affects community dynamics and ecosystem processes in tropical dry forest ecosystems initially limited by nitrogen (N) (A) or phosphorus (P) (B).

Our model includes the possibilities that there are multiple mechanisms that account for N- or P-limitation (top bars). Arrows represent hypothesized direction of change in pool or process under N deposition.

(Montano et al., 2007), N, and P (Galicia and Garcia-Oliva, 2004) are dependent upon season and the intensity of plant-microbe competition.

Mycorrhizal fungi deserve special mention. Mycorrhizae live in close symbiosis with plants and their responses to nutrient addition depend partially upon plant nutrient status as well as background nutrient limitation (Treseder, 2004). N deposition decreases arbuscular mycorrhizal biomass in both temperate and tropical forests (Johnson et al., 2003; Treseder, 2004; Camenzind et al., 2014; Krashevska et al., 2014; Wurzburger and Wright, 2015), but responses to N and P addition are context-dependent. Ecological relationships between plants and their mycorrhizae depend both upon soil C:N:P stoichiometry (Johnson et al., 2015) and mycorrhizal type (e.g., arbuscular, ectomycorrhizal, etc). Theory predicts that N deposition may lead to increased mycorrhizal biomass and more parasitic phenotypes under N-limitation and decreased biomass/more mutualistic phenotypes under P-limitation (Johnson et al., 2015). Finally, because mycorrhizae may enhance plant water uptake to different degrees depending on nutrient availability and mycorrhizal type (Auge, 2001, 2004; Lehto and Zwiazek, 2011), any change in mycorrhizal colonization or composition related to nutrient deposition may affect plant water relations during drought, potentially leading to differential feedbacks on evergreen and deciduous species in TDFs.

Communities: Plants

N deposition may reduce plant diversity in a number of ecosystems (Bobbink et al., 2010). While large-scale studies addressing this in TDFs are rare, a number of seedling pot experiments have tested how particular functional groups respond to increased nutrient availability (Lawrence, 2003). In general, higher N availability enhances biomass and relative growth rates and decreases root: shoot ratios (Huante et al., 1995a; Mendieta-Araica et al., 2013). The response to elevated nutrient availability is higher in light-demanding species compared to shade-tolerants (Huante et al., 1995a), in small-seeded species compared to those with larger seeds (Huante et al., 1995b), in slow-growing compared to fast-growing species (Khurana and Singh, 2004; Tripathi and Raghubanshi, 2014), and in non-legumes compared to legumes (Tripathi and Raghubanshi, 2014). However, not all of these generalizations are consistent among studies.

In field fertilization experiments in TDFs, seedling growth and survivorship in fertilized plots was higher than controls overall, but this effect was species- and site-specific (Salinas-Peba et al., 2014). Additionally, seedling responses tended to be higher for N vs. P addition (Salinas-Peba et al., 2014), suggesting that ANPP may be N-limited, at least for young plants. Fertilization of regenerating TDFs in Mexico led to higher seedling recruitment, survival and growth (Ceccon et al., 2003, 2004), increases in herbivory, and increased leaf P or N content in trees (in young and old sites, respectively) (Campo and Dirzo, 2003). In this experiment, responses to fertilization were higher in young forests with high light availability, but again, these depended on particular species (Ceccon et al., 2003).

Altered nutrient availability may also impact the intensity of interspecific competition, generating shifts in community composition over time. Six years of fertilizing an abandoned

TABLE 1 | List of processes, hypothesized changes under nitrogen deposition in tropical dry forests, and whether this change is supported by the literature, along with key references.

Process	Hypothesized response to nitrogen deposition	Support for hypothesis	Important mediating ecosystem state factors	Relevant literature
Microbial biomass	Increase in N-limited forest/ no change in P-limited forest	P addition increases microbial biomass in TDF more than N	Water availability, plant-microbe competition	Galicia and Garcia-Oliva, 2004; Montano et al., 2007; Waring et al., 2013; Li et al., 2015a
Microbial enzymes	Increase hydrolytic enzymes and decrease oxidative	Supported in wet tropical forests; **no data for TDF**	Nutrient limitation of microbial biomass	Cusack et al., 2010, 2011
Mycorrhizae	Biomass decreases in N-limited forests/increases in P limited systems	Supported in temperature and wet tropical forests /**no data for TDF**	Mycorrhizal type, underlying nutrient limitation	Johnson et al., 2003; Treseder, 2004; Camenzind et al., 2014; Wurzburger and Wright, 2015
Plant diversity	Decrease	Insufficient long-term data from TDF/seedling growth and survival vary with N and/or P addition	Light availability, species traits	Huante et al., 1995a,b; Ceccon et al., 2003, 2004; Khurana and Singh, 2004; Bobbink et al., 2010; Siddique et al., 2010; Tripathi and Raghubanshi, 2014
ANPP	Increase on N-limited soils/no change on P-limited soils	Data are equivocal; results often suggest P and N co-limitation and species-specific responses	Soil age, successional stage	Torres and Franco, 1994; Campo and Vázquez-Yanes, 2004; Campo et al., 2012
BNPP	Decrease	**No data for TDF/**in savanna, N addition did not change BNPP but PK increased it	Soil age, successional stage	Barger et al., 2002; Li et al., 2015b
Litter decomposition	Decrease	Supported in TDF/P addition increases decomposition and N decreases it	Rainfall regime, plant species	Campo et al., 2007; Powers and Salute, 2011; Anaya et al., 2012; Lv et al., 2014
Soil C stocks	Slow pools increase/fast pools decrease	No N effects but P addition decreased soil C in young TDF, increased soil C in old TDF/**no TDF data on soil C fractions**	Mineralogy, ANPP responses, rainfall regime	Gamboa et al., 2010; Bejarano et al., 2014b
Soil N transformations and N_2 fixation	Decrease N fixation, but accelerate N	Soil N pools and mineralization rates increased with N/**no TDF data for N_2 fixation**	Water availability, season, successional status	Erickson et al., 2002; Solís and Campo, 2004; Verma et al., 2013

No empirical data from tropical dry forests is indicated in bold.

pasture with N and P in a highly seasonal region in Amazonia favored the growth of a few nutrient-responsive early-successional tree species, decreasing tree assemblage evenness (Siddique et al., 2010). We hypothesize that higher nutrient availability could impact species composition through similar mechanisms in regenerating TDFs (**Figure 1**), though there is a dearth of long-term experimental data that can clarify community-wide impacts (**Table 1**).

Ecosystem Processes: Net Primary Production

Responses of NPP to added N or P indicate whether primary productivity is limited by N, P, or both (**Figure 1**). Fertilization experiments can also offer mechanistic insight by demonstrating how tree stem diameter growth, litterfall, and fine root production each respond to increases in nutrient availability. Fertilization experiments that measured ANPP in TDFs after nutrient addition have yielded varied results. A fertilization study in a plantation forest in Venezuela showed no effect of P on wood production (Torres and Franco, 1994), while other studies have demonstrated strong co-limitation of ANPP by N and P (Campo and Vázquez-Yanes, 2004). In the latter study, responses of litterfall to nutrient addition depended upon soil type, underscoring that leaf and wood production may respond differently to element availability. Complicating matters further, the experiment in Mexico also showed that diameter growth responses to added nutrients depended on species identity (Campo et al., 2012).

Belowground NPP results are similarly heterogeneous. A meta-analysis of fine root production across temperate and wet tropical ecosystems showed decreases in fine root production

with increases in N deposition, coupled with an increase in coarse root stocks (Li et al., 2015b). By contrast, a savannah fertilization experiment showed increased fine root production only in plots fertilized with P, and not with N (Barger et al., 2002). While these studies have done much to elucidate how N and P influence NPP, more detailed fertilization experiments in TDF are needed that address interactions between nutrient addition and factors that modify forest responses including succession, edaphic gradients, management, and water availability.

Ecosystem Processes: Decomposition

In general, decomposition rates are mediated by interactions among climate, litter quality, and decomposer communities (Meentemeyer, 1978; Kwabiah et al., 1999). Nutrient addition could affect decomposition via changes in litter chemistry or alterations to microbial community composition or function (Hobbie and Vitousek, 2000; Campo et al., 2012). However, within TDFs litterfall and soil nutrient availability are regulated by intra-annual precipitation events (Anaya et al., 2012), and as a result, decomposition may be primarily limited by water availability (Lambert et al., 1980; Read and Lawrence, 2006).

Plot-scale fertilization with N and/or P changed both litter nutrient concentrations and quantities in a TDF in Mexico, although the magnitude of change depended on forest successional age (Campo et al., 2007) and soil N availability (Campo et al., 2012). This is consistent with evidence that N addition effects depend on the form of added N (Lv et al., 2013), litter chemistry or quality (Kwabiah et al., 1999), and site-specific characteristics such as annual rainfall and soil nutrient availability (Bejarano et al., 2014a). In another fertilization experiment in a seasonal tropical forest in China, added P increased decomposition rates while the addition of N inhibited decomposition (Chen et al., 2013). Lab experiments also find that P and micronutrients accelerate leaf litter decay in TDF, while added N retards it (Powers and Salute, 2011). The mechanisms for why added N decreases decomposition, even in N-limited systems include both biotic and abiotic explanations, and this pattern appears robust across ecosystems (Treseder, 2008).

Ecosystem Processes: Soil Carbon Dynamics

Soils contain the largest terrestrial carbon stock, thus changes to the inputs, outputs, or turnover times of soil organic carbon (SOC) may affect atmospheric CO_2 concentrations and climate (Schlesinger, 1997). Much research has been devoted to understanding how N deposition affects SOC storage in temperate ecosystems (Liu and Greaver, 2010; Lu et al., 2011), and the mechanisms that mediate such changes (Li et al., 2015b). If increased N deposition stimulates plant productivity, inputs to the soil from leaf or root litter may increase. Furthermore, N deposition may also repress lignolytic enzyme activity, causing decreases in organic matter decay rates and enhancing SOC storage (Eisenlord et al., 2013). Alternatively, changes in nutrient availability may increase root exudation, "priming" the decomposition of organic matter and decreasing soil C storage (Phillips et al., 2011). Understanding the net effects of these processes is further complicated by the fact that SOC is composed of diverse compounds or fractions that vary in turnover times and mechanisms of stabilization (Sollins et al., 1996), and labile, slow, and stable SOC fractions may respond differently to N or P addition (Cusack et al., 2010; Nottingham et al., 2015).

In an experiment in a wet tropical forest, long-term N addition reduced labile SOC pools, corresponding to increased hydrolytic enzyme activity (Cusack et al., 2010, 2011). However, overall slow SOC pools (and thus total SOC) increased, as a function of reductions in oxidative enzymes (Cusack et al., 2010, 2011). By contrast, in a large-scale experiment in 10 and 60-year old regenerating TDF in Mexico, 3 years of P fertilization decreased total SOC in the young, N-limited forest, but increased it in the P-limited older forest, with no significant effects of N or N+P addition (Gamboa et al., 2010). Together with laboratory incubation experiments (Bejarano et al., 2014b), these studies emphasize that the response of SOC in TDFs to N deposition may be complex, and vary according to the nutrient limitation status of plants and soil microbial biomass.

Ecosystem Processes: Nitrogen Cycling

N_2 fixation, N mineralization, and (de)nitrification are all regulated by the availability of N. Evidence from wet forests suggests that legumes down-regulate symbiotic N_2 fixation as soil N becomes more available through secondary succession (Batterman et al., 2013; Sullivan et al., 2014a). Similarly, rates of free-living N_2 fixation in soils and the forest floor may decrease with increasing N availability, as seen in wet forests of Puerto Rico (Cusack et al., 2009). However, water availability may be a more important control on legume nodulation in TDFs (Gei and Powers, 2015), in which case N_2 fixation rates may change little with nutrient addition. A fertilization study in a TDF in India showed that N pools and mineralization rates increased with added N (Verma et al., 2013); however, both nitrogen cycling (Saynes et al., 2005) and nutrient addition effects on N cycling in another TDF in Mexico depended on season and forest successional status (Solís and Campo, 2004). Last, nitric and nitrous oxide emissions should increase under N deposition, particularly during the wet season (Erickson et al., 2002; Wang et al., 2014), and especially from N-saturated or P-limited forests (Hall and Matson, 1999).

Conclusions and Directions for Future Research

Taken together, the evidence on tropical dry forest responses to nutrient availability suggests several robust generalizations (**Table 1**). First, tree species respond individually to amendments with different nutrients, which likely reflects differences in life history strategies and functional traits. Second, TDF ecosystem responses to increased N or P are context-dependent and are

mediated by ecosystem state factors such as soil parent material, prior land-use history, species composition, and both intra- and interannual variation in water availability.

Our analysis also revealed large knowledge gaps that represent critical directions for future research (**Table 1**). A major uncertainty in TDF ecology is understanding the extent to which N, P, or other elements most limit productivity, the consequences of nutrient limitation under altered nutrient deposition regimes, and how belowground communities and processes respond (**Table 1**). Although many methods have been proposed to accomplish this (Sullivan et al., 2014b), the "gold standard" of ecosystem ecology remains large-scale fertilization experiments. Such experiments provide the opportunity to resolve nutrient addition effects and mechanisms across a hierarchy of scales from microbial to trees. Ideally, such experiments could be established along gradients of ecosystem state factors such as annual rainfall (Bejarano et al., 2014a) or forest age (Campo and Vázquez-Yanes, 2004), and in a greater diversity of dry forests including those in India and Africa, which are poorly represented in the literature. Most of the handful of field-scale fertilization experiments in TDF occurred in Mexico. Although these studies have provided valuable insights, establishing similar studies in a range of dry forests is critical if we are to advance our knowledge of TDF responses to global change (Fahey et al., 2015). Moreover, we need to look beyond N and P to the other elements that affect forest processes (Kaspari et al., 2009; Powers and Salute, 2011). Such knowledge is necessary for both accurately representing TDF dynamics in ecosystem simulation models and managing TDF under anthropogenic nutrient deposition.

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Amazon Forest Ecosystem Responses to Elevated Atmospheric CO_2 and Alterations in Nutrient Availability: Filling the Gaps with Model-Experiment Integration

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The impacts of elevated atmospheric CO_2 (eCO_2) and alterations in nutrient availability on the carbon (C) storage capacity and resilience of the Amazon forest remain highly uncertain. Carbon dynamics are controlled by multiple eco-physiological processes responding to environmental change, but we lack solid experimental evidence, hampering theory development and thus representation in ecosystem models. Here, we present two ecosystem-scale manipulation experiments, to be carried out in the Amazon, that examine tropical ecosystem responses to eCO_2 and alterations in nutrient availability and thus will elucidate the representation of crucial ecological processes by ecosystem models. We highlight current gaps in our understanding of tropical ecosystem responses to projected global changes in light of the eco-physiological assumptions considered by current ecosystem models. We conclude that a more detailed process-based representation of the spatial (e.g., soil type; plant functional type) and temporal (seasonal and inter-annual) variability of tropical forests is needed to enhance model predictions of ecosystem responses to projected global environmental change.

Keywords: Amazon, carbon allocation, elevated CO_2, free-air CO_2 enrichment (FACE), nutrient addition, tropical forest

INTRODUCTION

Tropical forests account for approximately one fourth of the total global forest carbon (C) stock (Phillips et al., 2009; Pan et al., 2011; Carvalhais et al., 2014). The Amazon basin represents the largest continuous region of tropical forests, storing about 150–200 Pg C (Feldpausch et al., 2012) and accounting for 14% of C fixed by photosynthesis in the terrestrial biosphere (Zhao and Running, 2010). A decrease or cessation of the Amazon forest C sink could create strong positive feedbacks on global climate change. It is therefore of paramount importance to enhance the mechanistic understanding of potential feedbacks between tropical C storage and projected global environmental change (Zhou et al., 2013; Zuidema et al., 2013).

Amazon forests appear vulnerable to increasing moisture stress, with the potential for large C losses to exert feedbacks on climate change (Phillips et al., 2009). Global circulation models project an increase of droughts affecting the Amazon region (Malhi et al., 2008) due to increasing frequency of climate anomalies associated to increasing sea surface temperatures (Lewis et al., 2011), which could lead to loss of Amazon forest further accelerating climate change (Rammig et al., 2010). Efforts to quantify the response of aboveground carbon storage derived from forest inventory plots concluded that such events have the potential to reverse a multi-decadal biomass C sink across Amazonia (Phillips et al., 2009). However, to date predicted responses of the Amazon forest to projected global climate changes (mainly precipitation and temperature) remain largely speculative due to a lack of direct experimental evidence (Malhi et al., 2009; Davidson et al., 2012; Cernusak et al., 2013). As a surrogate, ecosystem models that include the mechanistic representation of key ecosystem processes, such as photosynthesis, respiration, growth, and C allocation, have been used for projections of ecosystem responses to changes in climate, atmospheric CO_2 and nutrient availability (e.g., Goll et al., 2012; Huntingford et al., 2013; Joetzjer et al., 2014; Smith et al., 2014; Yang et al., 2014). However, due to differences in the representation of ecophysiological processes and their sensitivity to environmental stresses model simulations predict either dramatic Amazon forest dieback for the twenty first century due to changes in precipitation patterns (Cox et al., 2008) and rising temperatures (Cox et al., 2004), or simulate strong resilience of tropical forests due to a theoretically supposed CO_2 fertilization effect on primary productivity (Cox et al., 2013; Huntingford et al., 2013). As a consequence, climate-driven loss of tropical forest biomass could potentially be mitigated by CO_2 fertilization of forest productivity (Lapola et al., 2009; Rammig et al., 2010).

Nonetheless, lack of experimental evidence, in particular for the tropics, hinders the validation of current ecosystem models in predicting responses to increasing atmospheric CO_2 concentrations and alterations in nutrient availability. In turn, one of the main limitations of state-of-the-art models for reliably projecting potential changes in the Amazon C pool is the uncertainty associated with critical underlying ecosystem processes such as the physiological response of tropical trees to eCO_2 and nutrient limitation (Medlyn et al., 2015; Norby et al., 2016; Reed et al., 2015). So far, manipulative ecosystem experiments have predominantly been conducted in temperate and boreal regions. These experiments have greatly enhanced our understanding of forest ecosystem responses to eCO_2 and increasing nutrient inputs from anthropogenic sources (e.g., Rastetter and Shaver, 1992; Reich et al., 2014; Norby et al., 2016). For instance, free-air CO_2 enrichment (FACE) experiments in temperate areas indicate an initial increase in forest productivity (Norby et al., 2005), which is ultimately limited by soil nutrient availability (Norby et al., 2010). However, temperate and boreal regions are predominantly limited by soil nitrogen (N) availability, whereas lowland tropical forests are relatively rich in N, but characterized by highly weathered soils with low soil phosphorus (P) availability (Walker and Syers, 1976; Vitousek and Sanford, 1986; Quesada et al., 2012). Because of differences between tropical forests and temperate forests in biological complexity, nutrient cycling, and climate regimes, we expect differential responses of tropical forest ecosystems to eCO_2 and alterations in nutrient availability.

Ecosystem scale experiments subjecting mature forests to eCO_2 and nutrient addition in the tropics, and in particular the Amazon forest, are crucial for filling major gaps in our understanding of expected global change effects (Hickler et al., 2008; Cernusak et al., 2013; Norby et al., 2016). Two major endeavors are currently underway in Amazonia; the first free-air carbon enrichment experiment in a mature tropical old-growth forest (AmazonFACE; Lapola and Norby, 2014), as well as the first large-scale factorial nutrient addition experiment (AFEX; Amazon fertilization experiment). These experiments aim to assess the effects of eCO_2 and alterations in nutrient availability on biogeochemistry, ecology and resilience of the Amazon forest. Based on an assumption-centered integrated model-experiment approach (Medlyn et al., 2015), the goal of this paper is to discuss the plausibility and uncertainties of three hypotheses related to potential responses of tropical ecosystem processes to eCO_2 and alterations in nutrient availability: ***(1) Elevated CO_2 will increase C source activity (i.e., assimilation) and thus could stimulate C sink activity (i.e., growth) (2) Low nutrient (phosphorus) availability of tropical soils will limit C sink activity but may be alleviated by C allocation belowground; (3) Shifts in plant C allocation will increase C turnover and thus might decrease C storage in tropical forests.*** We explore each of these hypotheses in relation to current model assumptions and observational evidence (**Figure 1**).

ELEVATED CO_2 WILL INCREASE C SOURCE ACTIVITY (I.E., ASSIMILATION) AND THUS COULD STIMULATE C SINK ACTIVITY (I.E., GROWTH)

Current model formulations assume that tropical forests have the potential to respond more strongly to eCO_2 compared to temperate and boreal forests, based on the eco-physiological understanding of photosynthesis that is represented by a scheme introduced by Farquhar et al. (1980). Therefore most ecosystem models incorporating the Farquhar scheme (Long, 1991; Drake et al., 1997; Hickler et al., 2008, 2015; Cernusak et al., 2013) suggest that eCO_2 increases rates of leaf-level photosynthesis (Lloyd and Farquhar, 1996) based on the following simplified relationship:

$$A = min \left\{ \begin{array}{c} JL = f\,(PAR) \\ Jc = f\,(ci,\,T,\,\sim H_2O) \\ Je = f\,(T) \end{array} \right\}$$

where the rate of gross photosynthetic CO_2 assimilation (A) is determined by the rate of carboxylation (J_c), and the potential rate of electron transport (J_e) at a particular irradiance (J_L). Several factors may limit the rate of carboxylation; i.e., the relative partial pressure of CO_2 and O_2 (c_i), the amount of activated

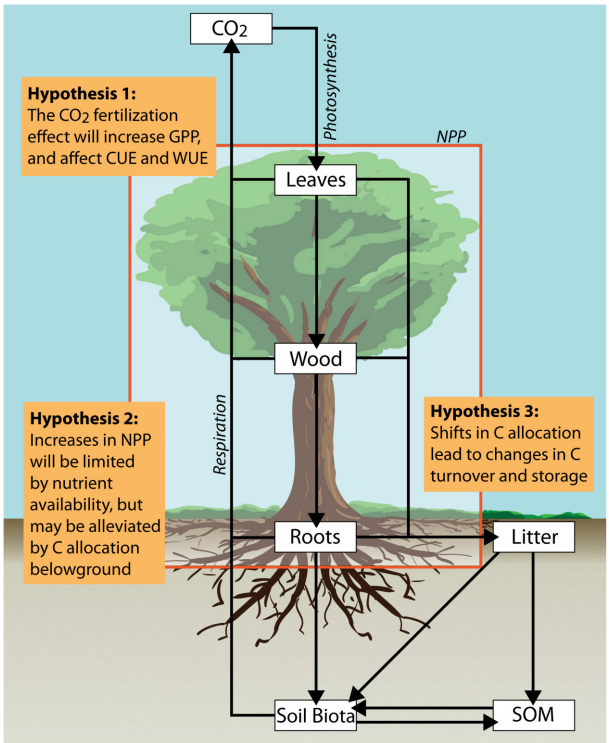

FIGURE 1 | Simplified conceptual model depicting major pools and fluxes of carbon in tropical forests. Assumption-based hypotheses to be targeted by the proposed Amazonian long-term ecosystem experiments are discussed in the text.

Rubisco enzyme, and the rate of acceptor regeneration (Farquhar et al., 1980). Due to the influence of c_i and J_L on the temperature (T) dependence of A, and the fact that under eCO_2 carboxylation dominates over oxygenation and increases with T until limited by the maximum rate of J_e, we might expect a stronger positive response of net photosynthesis to eCO_2 in warmer environments, such as tropical forests (Lloyd and Farquhar, 2008).

However, because carbon gain and water loss are regulated by the rate of leaf stomatal closure, a downregulation of stomatal conductance due to high vapor pressure deficit has the potential to substantially reduce the eCO_2-induced increase in leaf-level photosynthesis (Beedlow et al., 2008; Körner, 2009; Holtum and Winter, 2010). Based on the observed coupling between photosynthesis and stomatal conductance such effects are included in most ecosystem model formulations and therefore under eCO_2, most models simulate an increase in water-use efficiency (WUE; De Kauwe et al., 2013) i.e., the rate of photosynthesis to transpiration. However, studies based on carbon isotope fractionation in tropical tree rings suggest that increasing WUE does not necessarily trigger increased tree growth (Nock et al., 2011) and thus found no stimulation of tree growth in response to CO_2 fertilization (van der Sleen et al., 2014). This dilemma might result from the fact that under sub-optimal conditions enhanced "source activity" (i.e., assimilation) of leaves in response to eCO_2 might not stimulate increased "sink activity" (i.e., growth) of tropical trees due to other factors limiting plant growth (Körner, 2003). Highlighting the interactive effects of instantaneous biogeochemical processes that have to be considered in global ecosystem models this illustrates why an eCO_2-induced stimulation of photosynthetic C assimilation might not necessarily result in enhanced C storage of tropical forests in response to eCO_2 (Körner, 2003).

Recent research indicates that during drought trees prioritize growth by reducing autotrophic respiration that is unrelated to growth instead of reducing total productivity (Doughty et al., 2015a). This indicates that projected global changes such as increasing drought frequency and mean annual temperatures influence the ratio of photosynthesis to respiration (R_a) and thus affect plant carbon use-efficiency (CUE) i.e., the rate of net primary production (NPP) to gross primary production (GPP; Gifford, 2003; DeLucia et al., 2007). Similarly, in process-based models the optimum temperature for maximum net primary production (where NPP = GPP−R_a) depends on the balance between temperature effects on photosynthesis and respiration. However, there are still large uncertainties to which extent these physiological processes can acclimate in response to projected global changes (Huntingford et al., 2013). Both photosynthesis and respiration are speculated to be capable of dynamic thermal acclimation as a long-term temperature response. For instance, when acclimation is not considered in ecosystem models this usually leads to widespread forest dieback compared to when photosynthetic temperature acclimation is accounted for (Sitch et al., 2008). However, acclimation is challenging to incorporate in models in a mechanistic way because different species appear to vary in their acclimation strategies (Medlyn et al., 2011). Thus, the overall effect of global changes on tropical forests represents the integration of simultaneous responses of ecophysiological processes, such as those discussed above but also on phenology, allocation, turnover, and decomposition. Ultimately, by integrating this mechanistic understanding based on empirical evidence from experimental studies in next generation ecosystem models we will be able to scale-up plot-level measurements to ecosystem-scale responses under sub-optimal conditions of resource availability.

LOW NUTRIENT (PHOSPHORUS) AVAILABILITY OF TROPICAL SOILS WILL LIMIT C-SINK ACTIVITY BUT MAY BE ALLEVIATED BY C ALLOCATION BELOWGROUND

Plants need a broad range of nutrients (N, P) and micronutrients in a certain stoichiometry to maintain NPP, growth and normal functioning (e.g., Liebig, 1840; Sterner and Elser, 2002; Körner, 2003). Hence, we expect that potential initial increases in NPP will be limited by low nutrient availability of tropical soils (**Figure 1**). In tropical forests N is mainly stored in biomass, and rapidly recycled from organic matter; and in addition to N deposition, symbiotic N_2-fixing plants appear at a high abundance (Houlton et al., 2008; Hedin et al., 2009). Phosphorus inputs in contrast are mainly rock-derived; P availability decreases with weathering, and with increasing soil

age it becomes gradually transformed and bound in recalcitrant complexes (e.g., Walker and Syers, 1976; Vitousek et al., 2010). Hence, in old and highly weathered Amazonian soils P rather than N appears to limit plant growth. Moreover, P availability seems to exert an important control on N cycling (Quesada et al., 2010; Vitousek et al., 2010), but our understanding of the nature of P limitations and N:P interactions is still expanding (Vitousek et al., 2010; Lambers et al., 2015). Ecosystem C-N models integrating above- and belowground interactions, and including constraints of plant stoichiometry strongly improved the representation of eCO_2 responses of two temperate FACE experiments (Zaehle et al., 2014). There is growing recognition that P-dynamics and N:P interactions, particularly for the tropics, are poorly represented in ecosystem models (Wang et al., 2010; Yang et al., 2011; Goll et al., 2012; Powers et al., 2015; Reed et al., 2015) For example, when N and P availability are included in global ecosystem models NPP is reduced by up to 25%, potentially compensating eCO_2 effects and turning the terrestrial biosphere into a net C source by 2100 (Wieder et al., 2015).

Nutrient limitations might restrain plant productivity, and reduce the proposed C sink strength of Amazonian forests, as has been shown for other tropical forests (Kaspari et al., 2008; Wright et al., 2011; Lambers et al., 2015; Powers et al., 2015). To date, three stand-scale nutrient addition experiments conducted in high diversity lowland tropical forests, indicate co-limitations of several nutrients regulating key plant physiological and ecosystem processes (Mirmanto et al., 1999; Kaspari et al., 2008; Wright et al., 2011; Pasquini and Santiago, 2012; Alvarez-Clare et al., 2013). However, even in relatively P-rich soils in Central America (Cleveland et al., 2011) P additions tend to have the strongest impacts, increasing decomposition rates (Kaspari et al., 2008) and stimulating growth of trees in smaller size classes (Wright et al., 2011; Alvarez-Clare et al., 2013). We therefore expect soil P to be the key nutrient restraining responses to eCO_2, buffering the proposed C sink strength (Aragão et al., 2009; Cleveland et al., 2011; Baribault et al., 2012; Quesada et al., 2012).

FACE-experiments conducted in temperate forest ecosystems showed that under eCO_2 an initial increase in NPP by enhanced photosynthesis was allocated to fine root production and root exudation to overcome increasing N limitation (Körner et al., 2005; Iversen et al., 2008; Norby et al., 2010). The low P-availability in the Amazon, however, rather hints toward a gradual P-limitation, but similarly as in temperate forests, this may be alleviated by increasing C allocated belowground into root production, exudates, and to mycorrhizal or N-fixing symbionts (Finzi et al., 2007; Houlton et al., 2008; Iversen, 2010). While increased root biomass might promote C sequestration, higher labile C inputs into the rhizosphere will provide energy for microbes and change microbial community composition and turnover (Deng et al., 2016). Increased root exudation could stimulate enzyme (phosphatase) production (Nottingham et al., 2012; Spohn et al., 2013; Stone et al., 2013) and induce changes of soil pH in the close vicinity of fine roots (Lloyd et al., 2001; Jones et al., 2009; Lambers et al., 2015), and enhance decomposition of leaf or root litter or of older soil organic matter ("priming-effect"; Fontaine et al., 2003; Kuzyakov, 2010). This could enhance P availability for microbial and plant uptake, but could lead to CO_2 losses as autotrophic respiration (R_a), and enhance heterotrophic soil CO_2 efflux (R_{het}) due to higher microbial growth and activity (Fontaine et al., 2004; Blagodatskaya et al., 2010; Kelley et al., 2011). If this is the case, we predict that increases in soil respiration will decrease a likely C fertilization effect in tropical forests. Alternatively, eCO_2 could increase leaf longevity and alter litter quality (e.g., increase C:P and lignin content), which could reduce decomposition and buffer CO_2 losses by R_{het}. Hence, conducting a FACE, as well as a factorial nutrient addition experiment will increase our mechanistic understanding of N and P limitation in mature, old growth tropical forests (Cleveland et al., 2011), and allow improving model-assumptions to enhance predictions of tropical ecosystem responses to eCO_2 and alterations in nutrient availability.

SHIFTS IN PLANT C ALLOCATION WILL INCREASE C TURNOVER AND THUS MIGHT DECREASE C STORAGE IN TROPICAL FORESTS

Due to potentially increased NPP in response to eCO_2, and a subsequent relative investment into belowground compartments we expect a general shift in plant C allocation (**Figure 1**). Nonetheless, the allocation of NPP between plant compartments, as well as to carbohydrate reserves and export to symbionts is usually not accounted for in ecosystem models (Fatichi et al., 2014) or often represented by constant fractions:

$$\frac{dCi}{dt} = \alpha NPP - \frac{Ci}{\tau}$$

where Ci represents a given plant C pool (e.g., leaves, wood, fine roots), α is a parameter in percentage and τ the turnover rate of Ci. Such constant allocation schemes perform poorly when reproducing eCO_2 effects (De Kauwe et al., 2014). This is partly due to the fact that key processes such as carbon allocation to leaf, root and wood, plant mortality and soil carbon decomposition fluctuate over time and space (Rowland et al., 2014) and thus this variability should be represented in ecosystem models. Therefore, allocation schemes based on functional relationships among biomass fractions that vary with resource availability perform best in capturing field-based observations (De Kauwe et al., 2014).

Recent observations indicate that photosynthesis and plant carbon usage are temporally decoupled allowing C to be allocated when it is ecological beneficial, rather than when C is environmentally most available (Doughty et al., 2015b). Hence, seasonal reductions in wood NPP were found associated with carbon preferentially allocated to either root or canopy NPP during the dry season (Doughty et al., 2015b). Due to significant differences in turnover times of plant tissues (i.e., leaves, wood, fine roots) this suggests that projected increases in temperature and dry season length could strongly affect tropical C storage by shifting C allocation away from wood NPP (Hofhansl et al., 2015) and toward canopy and root NPP to alleviate drought-induced resource limitation (Doughty et al., 2014). As a result, tropical C storage will differ considerably depending on if C

is stored in long-lived wood or allocated to plant tissue with reduced lifespan (Körner et al., 2005). In accordance, global vegetation models diverge considerably between estimates based on the representation of C allocation and pool turnover patterns. Nonetheless, despite the fact that residence time of C was found to dominate the uncertainty in the response of terrestrial vegetation to eCO_2 (Friend et al., 2014), most models predict increasing C sequestration in both biomass and soils in response to eCO_2 (De Kauwe et al., 2014).

Over the last decades the Amazon C sink has been substantial (Pan et al., 2011), however, recent observations suggest that the sink strength is declining due to increasing tree turnover and mortality rates (Brienen et al., 2015). Therefore, a realistic scenario in line with long-term inventory data (Phillips et al., 1998, 2008) seems that global alterations in atmospheric CO_2-concentration and nutrient availability could have generated a more dynamic tropical forests (Körner, 2004). As a result, accelerated life cycles of tropical trees (Bugmann and Bigler, 2011) and associated increases in turnover times and decomposition rates have the potential to adversely affect the Amazonian C sink strength in the long-term (Brienen et al., 2015).

THE WAY FORWARD: MODEL-EXPERIMENT INTEGRATION

It is becoming increasingly evident that high diversity tropical forests, and in particular the Amazon, pose great challenges for global ecosystem models. Hence, the common use of 1 or 2 plant function types (PFT) with fixed parameterization and thus a common response to environmental change does not apply to highly diverse flora. Recent studies indicate that tropical C storage may be strongly determined by the hyperdominance (Fauset et al., 2015) and functional composition of tropical tree communities in association with different life-history strategies of tropical trees (Fauset et al., 2012). A CO_2 induced shift to shorter average tree life span could favor fast-growing tree species that invest in low-cost tissue, which could increase the turnover of C and thus decrease the C storage at the landscape scale (Phillips et al., 2009; Fauset et al., 2012; Cernusak et al., 2013). In accordance, simulations of nonrandom species loss for fast-growing species with low wood density increased C storage by 10%, whereas the loss of high statured slow-growing species decreased C stocks by over 30% (Bunker, 2005). This further highlights that impacts of climate change and eCO_2 on the Amazon forest could be more subtle than projected by a catastrophic dieback scenario, and already ongoing.

Recent studies evaluating the sensitivity of wood NPP to seasonal and interannual variations in climate showed that the growth response of tropical trees is associated to site-specific differences in drought sensitivity (Hofhansl et al., 2014), such that consistent with a hypothesized tradeoff between maximum potential growth rate and hydraulic safety the strength of the growth seasonality response among trees is significantly correlated to functional traits (Rowland et al., 2013). This suggests that changes in future climate seasonality could differentially affect the C sink strength of tropical forests due to local resource availability and tree species composition (Hofhansl et al., 2014). The recently developed set of ecosystem models based on plant-traits (van Bodegom et al., 2012; Pavlick et al., 2013; Fyllas et al., 2014; Sakschewski et al., 2015) has the potential to capture these changes, since differences in vegetation communities are depicted as a continuum, rather than discretely. Implementation of ecosystem models capable of incorporating more flexible C allocation schemes as well as trait-based parameterization could strongly improve model predictions (Franklin et al., 2012) to simulate the response of tropical forest ecosystems to climate anomalies and other short-term disturbances.

Overall, these observations indicate strong feedbacks of species specific and edaphic factors on ecosystem C storage (see Körner, 2009) and strongly suggest that the interaction between biodiversity and forest dynamics should be considered when making projections about the role of tropical forests in the global C cycle in a CO_2-rich future (see Körner, 2004). This highlights the importance of manipulative *in-situ* experiments that simulate projected future conditions in high diversity tropical forest ecosystems and can be used to improve model-based predictions. Although given the time horizon of the two experiments neither AmazonFACE nor AFEX might capture the change in forest species composition, undoubtedly both experiments provide unique opportunities to investigate which life-history strategies and functional traits will be favored or disfavored under eCO_2 and increased nutrient availability. Furthermore, AmazonFACE and AFEX, will provide crucial information on tropical C allocation schemes and the response to global changes by investigating allocation of C to structural and non-structural compounds such as the relative investment belowground to fine roots, associated mycorrhizae, and the rhizosphere as well as monitoring potential feedbacks on autotrophic and heterotrophic CO_2 efflux. To that end, these manipulative and long-term ecosystem scale experiments aim to assess how predicted increases in atmospheric CO_2 concentrations and alterations in resource availability will affect the growth response and thus the C sink strength of the Amazon forest ecosystem under projected global changes.

SYNTHESIS

Following an integrated model-experiment approach guiding the design of *in-situ* investigations of tropical forest ecosystem responses to eCO_2 and nutrient manipulation in the proposed ecosystem-scale manipulation experiments will enable us to test whether eCO_2 has the expected fertilization effect at organ-, plant,- and ecosystem-level. We attempt to enhance the mechanistic understanding of potential responses of highly productive Amazonian forests thriving on highly weathered soils, and investigate whether nutrient (P) limitation affects the proposed CO_2 fertilization effect. Specifically, we will investigate *in-situ* if nutrient limitation triggers belowground C allocation to root biomass, mycorrhizal symbionts, and into the rhizosphere in exchange for nutrients, or stimulates microbial decomposition enhancing P recycling. By conducting the proposed long-term *in-situ* ecosystem manipulation experiments we aim to resolve

whether projected increases in atmospheric CO_2 concentration and alterations in nutrient availability will induce shifts in plant C allocation which could in turn increase C turnover, and decrease the long-term C storage capacity of the Amazon forest ecosystem. Generating this novel process-based understanding will further improve model-based predictions that can be used to upscale mechanistic principles to larger spatial scales.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial contributions to the manuscript. FH, KA, KF, LF and OV wrote the paper; KS designed the conceptual scheme; AR and DL conceived the analysis and contributed valuable comments on earlier manuscript versions.

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