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FOREST RESPONSES TO GLOBAL CHANGE DRIVERS: INSIGHTS FROM TEMPERATE AND TROPICAL ECOSYSTEMS

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A Nonna Pier(in)a,

vissuta in un periodo storico

in cui studiare, per le donne, era un privilegio.

Oggi direbbe "'Un ti avrei dato una Lira" ,

eppure mi ha dato tutto l'amore del mondo.

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Abstract

Forests, often likened to the lungs of our planet, are at the forefront of climate mitigation strategies. Understanding how major changes affecting the atmosphere (i.e., climate and pollutants) affect ecological and ecophysiological processes underpinning forest functioning is paramount to the preservation of the essential ecosystem services they provide. In the context of the ongoing era of global change, this thesis encompasses three distinct scientific studies that together contribute to advancing our understanding of the intricate ecophysiological responses of both temperate and tropical forest ecosystems to diverse global change drivers.

The initial two papers, "Effects of simulated nitrogen deposition on beech forests in Italy" and "Forest nitrogen dynamics in response to increasing nitrogen deposition: comparing above canopy and soil fertilization in a mature beech forest," focus on the impact of atmospheric nitrogen (N) deposition on two temperate forest ecosystems. These studies employ a long-term manipulative experiment, where the application of N fertilizer simulates the increase in N deposition. The first study investigates forest health under elevated N deposition by employing foliar nutrient and photosynthetic pigment concentrations as stress indicators. The second paper integrates measurements of N concentration and its isotopic composition across various forest compartments, coupled with molecular analyses of soil nitrogen functional genes, to characterize key processes underpinning the ecosystem N cycle. Furthermore, it compares the efficiencies of below and above-canopy fertilization treatments in simulating the natural N deposition phenomenon.

The third paper, "Multiple stable isotope approach in Tropical Montane Cloud Forests," delves into water and nutrient balances in a tropical montane cloud forest (TMCF). It underscores the imperative to address the knowledge gap on tropical forest ecosystems, which may be significantly compromised by human-induced climate change, such as alterations in precipitation water regimes and rising temperatures. This study utilizes carbon (C) and N stable isotopes in soil and plant tissues to evaluate water-use efficiency and nutrient dynamics, highlighting the potential vulnerability of TMCFs to evolving climatic conditions.

Collectively, these three studies articulate the challenges faced by forest ecosystems. From the nuanced effects of N deposition on foliar nutrient concentrations and intricate ecosystem N dynamics in mature deciduous forests to the vulnerable ecohydrological balance in the highly diverse tropical montane cloud forests, these insights deepen our comprehension of the complex interplay between human-induced changes and the resilience of these natural ecosystems.

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1. General Introduction

Forest ecosystems play a pivotal role in the global carbon (C) cycle, providing essential services to the environment and society. In the current era of global change, their significance is further underscored by their ability to sequester - through photosynthesis - the excess of atmospheric carbon dioxide (CO_2) derived from fossil fuel combustion. Covering approximately 30% of the world's land surface (FAO 2021), forests are estimated to sequester around 2.3 Pg C annually, 45% of terrestrial C (Bonan, 2008; Pan et al., 2011). Recognized as the natural "lungs of the Earth," forests contribute significantly to mitigating the impacts of climate change. However, their crucial role is constrained by their vulnerability to an array of global change drivers, resulting in a complex interplay between mitigation potential (contrasting climate change) and susceptibility (being affected by it).

Amid anthropogenic factors driving global change, the increase in atmospheric CO_2 and other greenhouse gases has induced climate alterations. These changes not only involve an anticipated global temperature rise of 2.2° - 2.7°C by 2100 (IPCC, 2022) but also an increase in the frequency and intensity of extreme climate events, including heatwaves, droughts, floods, and wind storms (IPCC, 2022). These events may trigger cascading impacts on both forest ecosystems and societal sustainability (Reichstein et al., 2021). Beyond climate change, other global change factors, such as nitrogen (N) deposition, biotic invasions, and land-use modifications, collectively contribute to altering the environmental conditions to which forests have slowly adapted. These factors have profound and intricate interactions, influencing forest health and productivity (Augustaitis & Bytnerowicz, 2008; Kozlov et al., 2009). While forests possess some adaptive capacity to disturbance, the rapid pace of global change factors outstrips the evolutionary adaptation processes (Steffen et al., 2006; Trumbore et al., 2015).

One important component of the global change discussed in this thesis is the increase in N deposition, recognized as a significant force impacting the health and growth of forest ecosystems, particularly in the case of temperate and boreal forests in the Northern Hemisphere (Etzold et al., 2020). Atmospheric N deposition involves various N compounds, mainly in the oxidized (NO_x) and reduced (NH_x) forms, emitted in the atmosphere from natural or anthropogenic sources and deposited back into forest ecosystems through wet or dry deposition (Tørseth et al., 2012). Despite a recent global analysis indicating a decreasing trend in total annual NO_x emissions in 2013, the persistence of high ammonia (NH_3) deposition raises concerns, making N deposition an ongoing issue impacting forest ecosystems with open questions regarding its magnitude and consequences (Huang et al., 2017; De Marco et al., 2021).

N deposition can function as both a benefit and a stressor for trees (Duan et al., 2016; Oksanen & Kontunen-Soppela, 2021) depending on whether there is too much or too little of nitrogen in the system (Galloway et al., 2003; Sutton et al., 2011). On one hand, increased N availability in N-limited ecosystems can enhance C assimilation (Fernandez-Martinex et al., 2014). In fact, a recent study has indicated that N deposition has led to an increase of 0.41 Pg C annually in the net primary productivity of global forests (Du and Vries, 2018). Moreover, stomatal conductance might be reduced by increases in N deposition (Cramer et al., 2009; Livingston et al., 1999), which may cause a decrease in transpiration and a potential higher tree water-use efficiency (WUE). WUE is the measure of the trade-off between C gain and water loss in trees and is a key parameter for understanding the metabolism of C and water-based economics of forest ecosystems (Keenan et al., 2013). Thus, N deposition can potentially change N availability in ecosystems and regulate C, N, and water cycles, including ecosystem WUE (Lu et al., 2019). On the other hand, excess N inputs to natural ecosystems may have detrimental effects on forests health. For instance, elevated N deposition, passing through the canopy, can directly damage plant leaves, while deposition to the forest floor contributes to acidification, eutrophication, and N saturation of forest ecosystems (Aber et al., 1998; de Vries, 2021). Furthermore, N deposition's impact extends to the mycorrhizal association of trees, biodiversity of sensitive species like lichens, and to an imbalance of nutrient ratios, such as N:P ratio (Lilleskov et al., 2019; Giordani et al., 2014; Sardans et al., 2016).

In Europe, the surge in forest productivity observed in recent years (Etzoled et al., 2020), attributed to elevated atmospheric CO_2, N deposition, and extended growing seasons, particularly in the northern regions, has increased the demand for nutrients by plants (Jonard et al., 2015). However, the existing nutrient content in the soil often falls short of meeting this escalating demand, resulting in a widespread decline in the mineral nutrient status of forests across Europe (Jonard et al., 2015). Indeed, plants rely on a minimum of 17 chemical elements throughout their life cycle, essential not only for growth but also for supporting various vital plant functions. Phosphorus (P), a pivotal element, plays a crucial role in numerous plant metabolic processes, including energy provision for metabolism (ATP), nucleic acid and membrane synthesis, photosynthesis, respiration, nitrogen fixation, and enzyme regulation (Raghothama, 1999; Talkner et al., 2015). Among macronutrients, N and phosphorus P most commonly limit primary production (Vitousek et al., 2010). P deficiency can lead to diminished C allocation to plant reproduction organs (Mengel & Kirkby, 1982). Potassium (K), abundant in leaves, significantly influences plant water-use efficiency (Tripler et al., 2006; Sardans et al., 2012; Sardans & Penuelas, 2015). Calcium (Ca) is crucial for synthesizing cell structures and enhancing plant resistance to drought and cold-induced stresses

(White & Broadley, 2003). Magnesium (Mg), situated at the core of chlorophylls, plays a vital role in plant photosynthetic activity (Hermans et al., 2004). Manganese (Mn) and iron (Fe), essential for enzyme synthesis, can become toxic in large quantities in acidified soils (Aerts & Chapin, 2000; He et al., 2016; Lynch & St Clair, 2004; Tian & Niu, 2015). N deposition, while stimulating plant growth by increasing N concentrations, concurrently leads to the dilution of other nutrient concentrations within plant tissues, causing a substantial shift in stoichiometric ratios and an alteration of nutritional balances (Tian et al., 2018). Additionally, N deposition may trigger soil acidification, changing nutrient availability and directly impacting plant nutrient uptake, further modifying stoichiometric ratios (Han et al., 2011). Indeed, in acidic soils, the availability of cation exchange bases (e.g., K^+, Ca^{2+}, and Mg^{2+}) diminishes, while concentrations of Mn^{2+}, Al^{3+}, and Fe^{3+} in the soil solution increase, potentially reaching toxic levels for plants (Bowman et al., 2008; Lynch & St Clair, 2004; Kaspari et al., 2008; Tian & Niu, 2015; Tian et al., 2016). These changes further contribute to the disruption of plant nutritional balances. However, despite numerous studies conducted on plant nutrition, knowledge regarding the effect of nitrogen abundance on nutritional balances in forest ecosystems is still lacking.

When moving from temperature to tropical regions, other factors may undermine forest health and functioning. Tropical forests, contributing approximately one-third of Earth's terrestrial gross primary productivity and half of the terrestrial C stored in vegetation (Lewis et al., 2015), wield significant global influence on global C cycle. Minor biome-wide alterations in tree growth and mortality can yield widespread consequences, either mitigating or exacerbating the rise in atmospheric CO_2 (Hubau et al., 2020). Notably, the annual net C gain in Amazonian forests is rapidly decreasing, with projections estimating that it will reach zero by 2035 (Hubau et al., 2020). The robustness of tropical C sinks is intricately linked to future CO_2 emissions (Meinshausen et al., 2011), leading to temperature elevation and hydrological shifts (Hubau et al., 2020).

In this scenario, tropical montane cloud forests (TMCFs) emerge as particularly susceptible to global change drivers, encompassing land use transformations, rising temperatures, and altered water regimes, all of which pose substantial threats to the integrity and resilience of TMCF ecosystems (Dietterich et al., 2022). These intricate ecosystems heavily rely on regular cycles of cloud and mist formation (Bruijnzeel, 2005; Whitmore, 1998), and any shifts in these dynamics may result in heightened tree mortality, alterations in species distribution, and modifications in stream dynamics, thus compromising the ability of TMCFs to provide essential hydrological services (Bittencourt et al., 2019; Bittencourt, 2014; Martin & Bellingham, 2016).

The tools for predicting ecosystem responses involve modeling approaches and filed observation under ambient or controlled conditions. In the latter case, manipulation experiments have been established, simulating changes in crucial environmental parameters like atmospheric CO_2 concentration, temperature, rainfall patterns, and the deposition of N compounds as projected by the Intergovernmental Panel on Climate Change (IPCC) scenarios for the next 50 to 100 years. Dynamic Global Vegetation Models (DGVM), representing the functioning of terrestrial biomes, offer insights into the potential impacts of climate change on forest ecosystems (Cramer et al., 2001). Forecasts from these models suggest that net primary productivity (NPP) and net ecosystem productivity (NEP) will peak in the mid-21st century before declining due to the combined influence of rising CO_2 and temperatures. Given the high uncertainty associated with these models, these predictions serve as indicative estimates requiring validation through long-term field experiments. Over time, technologies for environmental manipulation have advanced, aiming to make experimental sites as faithful as possible to natural forest growth conditions. To date, manipulative experiment data are predominantly derived from temperate and boreal forests in North America and Europe. However, such data are more constrained for primary tropical forests (De Marco et al., 2022), where the intricate nature of ecosystems and the absence of infrastructure have posed challenges to conducting field research, particularly in the recent past. Examples of manipulative experiments range from ventilated chambers (both open and closed) incorporating trees or ecosystem segments to Free Air CO_2 Enrichment (FACE) systems (Norby et al., 2016), manipulations of water inputs, controlled heating of entire plant communities or specific sections (Meromy et al., 2015, Rowland et al., 2015), and increased N deposition on ecosystem levels (see NITREX project, Tietema et al., 1995). Italy hosted various ecological research sites deliberately subjecting complete natural ecosystems to experimentally tailored environmental conditions. Notable projects included EURO/POPFACE, which explored the effects of elevated atmospheric CO_2 on biomass poplar plantation production (Marinari et al, 2007); the MIND project, which studied water availability effects on productivity and the C cycle in Mediterranean ecosystems (Alberti et al., 2007); the VULCAN project, to simulate temperature increase and drought impacts on Mediterranean shrubland; and OZONIT, to investigate the effects of ozone on vegetation (Scarascia-Mugnozza, 2006); the N deposition has been simulated in various mature deciduous forest ecosystems across Italy, both applying fertilizer directly to the forest floor and to the canopy (see for instance Da Ros et al., 2023; Teglia et al., 2022).

This thesis presents three distinct, yet complementary, studies that contribute to advancing our understanding on the intricate ecophysiological responses of both temperate and tropical forest ecosystems to global change drivers. The first two papers, titled "Effects of simulated nitrogen deposition on beech forests in Italy" and "Forest nitrogen dynamics in response to increasing nitrogen deposition: comparing above canopy and soil fertilization in a mature beech forest," focused on the impact of atmospheric nitrogen (N) deposition on two temperate forest ecosystems. These studies employ a long-term manipulative experiment wherein the application of N fertilizer simulates the intensification of N deposition. The first research assesses forest health under elevated N deposition by using foliar nutrient and photosynthetic pigment concentrations as stress indicators. The second paper employs integrated measurements of N concentration and its isotopic composition across various forest compartments, along with molecular analyses of soil nitrogen functional genes, to characterize key processes underpinning the ecosystem N cycle. Additionally, it compares the efficiencies of below and above-canopy fertilization treatments in simulating the natural N deposition phenomenon. The third paper, titled "Multiple stable isotope approach in Tropical Montane Cloud Forests," delves into water and nutrient relationships in a tropical montane cloud forest (TMCF). It underscores the necessity of addressing the knowledge gap on tropical forest ecosystems, which may be significantly compromised by human-induced climate change, such as alterations in precipitation water regimes and rising temperatures. This study employs carbon (C) and N stable isotopes in soil and plant tissues to assess water-use efficiency and nutrient dynamics, shedding light on the potential vulnerability of TMCFs under rapid changes in climatic conditions.

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2. Effects of simulated nitrogen deposition on the nutritional and physiological status of beech forests at two climatic contrasting sites in Italy

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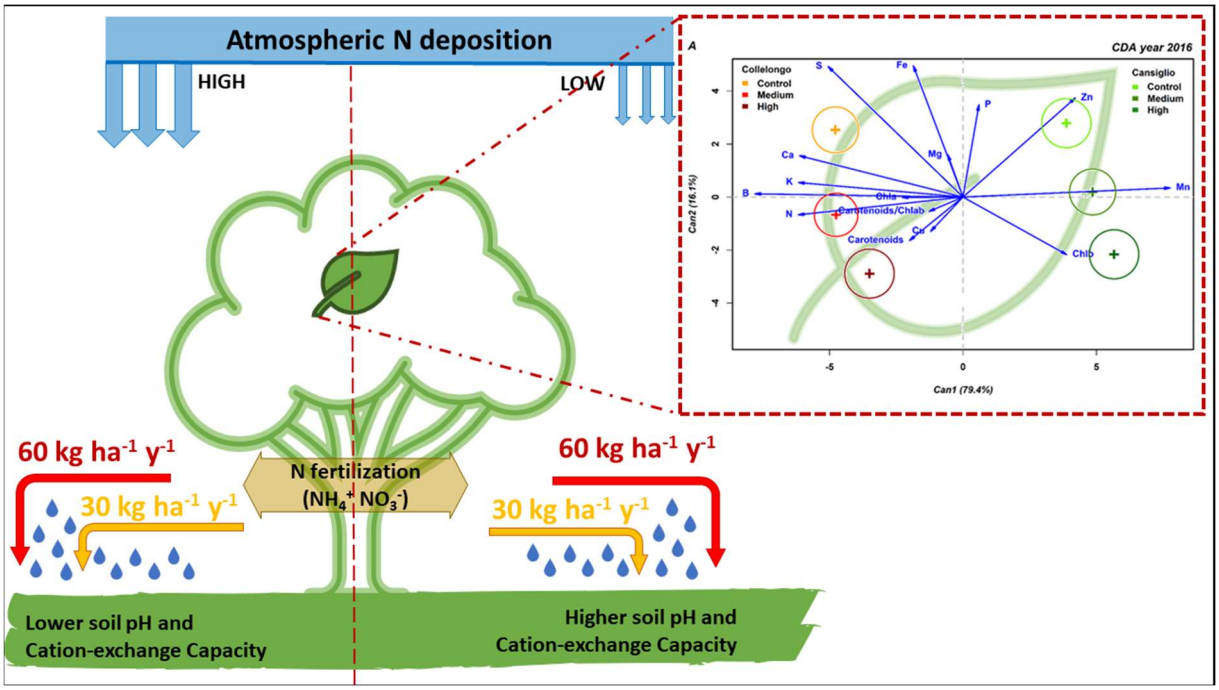
2.1. Summary

Anthropogenic activities have resulted in a significant increase of reactive nitrogen (N) compounds in the atmosphere and a rise in N deposition on forest ecosystems. Increasing N loads impact forest productivity and health, altering tree physiological status and nutrient balance with possible beneficial and detrimental consequences. The impact of N deposition has received considerable attention by scientific research, covering medium and high latitudes, while experimental studies in the Mediterranean area are almost absent. The present study used a manipulative approach, through replicated N additions (background deposition, 30, 60 kg N ha^{-1} yr^{-1}) to simulate the cumulative effects of N deposition in two beech (Fagus sylvatica L.) forests located in contrasting climatic regions of Italy. Leaf nutrients and photosynthetic pigments were tested as monitoring indicators after four years of N fertilization. Foliar N and pigment concentrations indicated not limiting N conditions at both forest sites, although changes in chlorophylls and carotenoids showed an early response of the canopy to N additions. N-to-phosphorus (P) and sulfur (S) ratios increased under elevated N fertilization, which could be partly related to the relative enhancement of foliar N concentration, and partly associated with the reduction of foliar P and S. The two eutrophic beech forests monitored were not severely affected by chronic N addition, not showing critical nutritional and physiological impairments over the short to medium period. However, the modifications in leaf nutrient and pigment compositions showed an incipient stress response and accentuated the differences induced by climatic and soil characteristics at the two sites. The potential use of nutrients and photosynthetic pigments in monitoring forest N deposition under contrasting climatic conditions and the eventual limits of manipulative experiments are discussed.

2.1. Graphical abstract



2.2. Introduction

Human activities over the last decades have drastically altered the natural nitrogen (N) cycle, as fertilizer manufacturing and fossil-fuel combustion have generated and released in the atmosphere an increasing amount of N reactive compounds such as nitric oxides (NO_x) and ammonia (NH_3), which has then deposited over managed and natural ecosystems.

Sulfur (S) deposition, often associated with N deposition in the past, has now dropped dramatically due to environmental regulations (Berger et al., 2016; Cecchini et al., 2021; Johnson et al., 2018), bringing N deposition to the forefront of current environmental concerns. Indeed, atmospheric N deposition to forests has reached globally 187 million tonnes in 2005 and has been estimated to almost double by 2050 (Galloway et al., 2008; Law, 2013).

With these concerns in mind, a Europe-wide monitoring scheme was set up in 1985 as part of the UN Convention on Long-range Transboundary Air Pollution (CLRTAP); the International Cooperative Programme on Assessment and Monitoring of Air Pollution Effects on Forests (ICP Forests) ensures the long-term monitoring of forest conditions and, through more than 800 Level 2 intensive study plots, periodic measurements of atmospheric N deposition, N and nutrient concentrations in vegetation and soils. In Italy, in particular, northern ICP Forests sites have been subjected to the highest levels of throughfall N deposition (up to a maximum of 28.8 kg N ha^{-1} y^{-1}), whereas the lowest rates have been recorded at southern sites (4.5 kg N ha^{-1} y^{-1}) (Ferretti et al., 2014).

N deposition appears to have had important effects on tree nutrition. The evaluation of the foliar data collected from the ICP Forests plots over a 20-year period has highlighted a critical decreasing trend in the foliar concentrations of some nutrients (Jonard et al., 2014). In particular, over the last 10 years a significant decline in foliar phosphorus (P), magnesium (Mg) and calcium (Ca) concentrations has been observed in beech (*Fagus sylvatica* L.) forests across Europe, while the foliar contents of N and S have shown a significant increase (Jonard et al., 2014). This trend has often led to unbalanced foliar nutrient ratios in forest trees, with a consistent increase of N:P ratios across all species in Europe (Penuelas et al., 2020; Talkner et al., 2015). In many European beech stands, in particular, an excess of N nutrition has been detected (Jonard et al., 2014), raising the concern that these forests could be exposed to the physiological and growth impairments often found in N saturated ecosystems (Aber et al., 1998).

N deposition can affect plant growth and carbon (C) sequestration by altering both nutrients and water limitations (Braun et al., 2020; Etzold et al., 2020; Sardans et al., 2012; Solberg et al., 2009). While the increasing N availability can reduce plant productivity under some conditions, in other

cases it has led to higher photosynthetic rates and plant growth (Solberg et al., 2009; Zhang et al., 2015). However, plant physiological response in leaf composition and stoichiometry to increasing atmospheric N is far from clear, especially in natural ecosystems. The leaf content of photosynthetic pigments, together with foliar nutrients, reflects the plant's light capture performance and, consequently, its functional ecology and productivity (Poorter et al., 2009; Scartazza et al., 2016). Chlorophylls and carotenoids play essential functional and protective roles in plant photosynthesis processes, and therefore they are often used as biomarkers in monitoring forest health conditions at different scales, from the leaf to the canopy level (Nestola et al., 2018; Niinemets et al., 2015; Scartazza et al., 2016).

While the long-term monitoring is invaluable to capture the status of European forests, it may fail to provide a detailed understanding of the role of different drivers involved in the long-term dynamics, such as increasing CO_2 and temperatures, N deposition, not to mention forest aging. Therefore, replicated manipulative studies should complement long-term monitoring by simulating N deposition under field conditions through long-term forest N fertilization to disentangle the effects of atmospheric N deposition alone. Although several studies have been carried out over the last decades, they have mainly focused on coniferous forests (de Schrijver et al., 2011; Hyvönen et al., 2008), for which fertilization has also been proposed for productive purposes under boreal and temperate climates (e.g., Billow et al., 1994; Kaakinen et al., 2009). However, different climatic and ecosystem conditions can significantly alter the N addition effects, and more limited information is available for forests of the Mediterranean area, where summer drought could constrain soil processes, plant function, and growth irrespective of N availability.

With this in mind, a replicated long-term N manipulation study was set up in two beech forests close to ICP forest Level 2 permanent monitoring plots historically exposed to different N depositions levels and located at two contrasting geographical sites (Cansiglio and Collelongo, Northern and Central Italy, respectively), to highlight the role of atmospheric N deposition in the changes detected as part of the ICP Forests monitoring. In Italy, beech forests are broadly distributed and represent about 12% of the national forest coverage, extending from the Alps to Sicily's southernmost sites. In the last decades, climate change and excess N deposition consistently threatened European beech forests' nutritional status (Braun et al., 2020; Jonard et al., 2014; Talkner et al., 2015). Due to Italian beech forests' environmental and socio-economic relevance, concern has grown about their sensitivity to climate change and chronic N deposition (Bonanomi et al, 2018; Cullotta et al., 2016; Valsecchi et al, 2008).

The present work, in particular, focuses on changes in leaf biochemistry at Cansiglio and Collelongo beech forests in response to four years of N additions. Leaf chemistry parameters, such as the content of elements and photosynthetic pigments, are a valuable tool to assess nutrient limitations status and health of plants, commonly adopted in nutrients manipulative experiments designed to establish the effects of fertilizations in forest ecosystems (Agren, 2008; Sullivan et al., 2014). Indeed, foliar biochemistry and elemental stoichiometry can appropriately reflect the availability of nutrients in the forests' soil (Cleveland et al.; 2011Han et al., 2005). This kind of leaves traits responds over a relatively short timescale to natural deposition or fertilization compared to tree growth and biometric parameters that often require more than a decade of treatments to manifest a shift in net primary production (Wright et al., 2011). The central questions explored in this work are: i) if N fertilizations have effects on forest trees' performance in terms of leaf nutrients content and photosynthetic pigments; ii) how N addition modifies the beech physiological response in two different climatic and environmental conditions.

2.3. Material and methods

2.3.1. Site description

Table 1. General description of Cansiglio and Collelongo experimental sites. Stand structural data from July 2015; N deposition data are an average for the period 1998-2017; climatic data are an average for the period 1981-2010. Total N soil con refers to mineral horizon A.

	Unit	Cansiglio	Collelongo
Latitude		46° 03' 29" N	41° 50' 58" N
Longitude		12° 22' 45" E	13° 35' 17" E
Elevation	m a.s.l.	1100	1560
Management		High forest	Coppice converted to high forest since 1950
Tree species		*Fagus sylvatica* L.	*Fagus sylvatica* L.
Stand age	years	130-140	105-125
Stand density	trees ha^{-1}	168	740
Mean tree DBH	cm	45	27
Mean tree height	m	29	21
FAO soil type		Haplic Luvisol	Humic Alisol
Annual precipitation	mm y^{-1}	1767	877
Mean annual temperature	°C	7.2	7.4
Mean annual total N deposition (open field) *	kg N ha^{-1} y^{-1}	17.7	5.99
Mean annual total N deposition (throughfall) *	kg N ha^{-1} y^{-1}	12.6	5.58
Total soil N concentration	%	0.34	0.67
Soil field capacity	% volume	21	14
Soil net N mineralization**	kg N ha^{-1} y^{-1}	95	120
Soil pH (at 20 cm depth)		4.3	5.6

* Cecchini et al., 2021. **In 2015, at the beginning of the experimental study.

The study was carried out in two Italian beech forests located on the Eastern Alps (Cansiglio; 46° 3' 19'' N 12° 22' 51'' E) and on the Central Apennines (Collelongo; 41° 50' 59'' N 13° 35' 6'' E). Both experimental sites are close to ICP Forest Level 2 permanent monitoring plots (VEN1 - Pian del Cansiglio, Northern Italy and ABR1 - Selva Piana-Collelongo, Central Italy, respectively; Marchetto et al., 2014). The vicinity of the two sites to long-term ICP monitoring stations (1996 to present) allowed the availability of key meteorological variables, forest growth rates, atmospheric N deposition data, leaf and soil biochemical characteristics (Table S1 and Fig. S1). This has made it possible to place the results of the present N fertilization study in a broader perspective.

At both sites, the forest is a pure single-layer beech stand; the stands were selected to have similar age and geological substrate, but with marked differences in climate and background N deposition. The Cansiglio area, at an elevation of 1150 m a.s.l., is characterized by the presence of an oceanic climate, with a mean annual temperature of 7.5 °C and maximum and minimum monthly temperatures of 22.4 and -5.7 °C, respectively. Annual precipitation is 1767 mm yr^{-1}, and a period of summer dryness is generally absent (Fig. S2). At the site, the atmospheric N deposition amounts, on average, to 17.7kg N ha^{-1} y^{-1} (Table 1, open field data; Cecchini et al., 2021) and exceeds the critical N load estimated by Ferretti et al. (2014). The forest grows on a soil classified as Haplic Luvisol (FAO 2006), developed on a calcareous pedogenetic substrate. The topsoil has an acid reaction, with pH values of 4.3 down to 20 cm depth; soil depth varies from 60 to 110 cm (Rezaie et al., 2018). In 2015 soil net N mineralization in the study plots amounted to 95 kg N ha^{-1} yr^{-1}. The beech forest structure at Cansiglio reflects its history of silvicultural management as a high forest, with a tree age of 130-140 years. Mean diameter at breast height (DBH) and height are 45 cm and 29 m, respectively. The density of the stand is about 170 trees per hectare (Table 1).

The experimental site of Collelongo, at an elevation of 1500 m a.s.l., lies close to a long-term eddy-covariance site. The stand environmental and structural conditions are representative of Central Apennine beech forests and reflect its silvicultural history and the typical Mediterranean-mountain climate (Scartazza et al., 2016). Mean annual temperature is 7.9 °C, with a monthly maximum and minimum temperature of 22.7 and -4.4 °C, respectively. The average annual rainfall at the site is 877 mm yr^{-1}, with a mild summer drought typical of the Mediterranean climate. Atmospheric N deposition at the site amount to 5.99 kg N ha^{-1} yr^{-1}. The soil is classified as Humic Alisols (FAO 2006), on a calcareous substrate, with sizeable skeleton and clay components and a pH around 5. Soil depth is variable and ranges between 40 and 100 cm. At the beginning of the experimental study, soil net N mineralization was 120 kg N ha^{-1} yr^{-1}.

The Collelongo beech forest has a composite structure stemming from its conversion from coppice into high forest, with ages ranging between 105 and 125 years at the study site. The forest still maintains a high and variable density, ranging from 800 to 3000 stems per hectare (1221 stems ha^{-1} with a DBH > 10 cm). Mean DBH and height are 19 cm and 21 m, respectively. The main characteristics of the two stands are summarized in Table 1.

2.3.2. Experimental design and N treatments

Three N addition treatments were compared at each site: untreated control (Control), soil addition of 30 (Medium) and of 60 kg N ha^{-1} yr^{-1} (High). The experiment was arranged in a completely randomized design with three replicate plots for each treatment, located at a suitable distance from ICP monitoring sites. The plots have dimensions of 30 x 30 m and are adjacent to each other. In order to avoid any lateral contamination effects, measurements were carried out on plants ($n \geq 3$) in the central area of each plot (15 x 15 m). Nitrogen fertilization started in June 2015 and then carried out in three annual applications (June, July, and September) over the following years, simultaneously at the two sites.

N was applied as ammonium nitrate (NH_4NO_3) and distributed on the ground in water solution using a backpack sprayer. Because of the small amount of solution added (< 1 mm yr^{-1}), no water was added to control plots.

2.3.3. Sample collection and analysis

Leaf samples were collected in 2016 and 2018 at the peak of the growth season (late July / early August) at both sites by the tree-climbing technique. Two branches developed in full light were sampled from 3 plants per plot. Leaves were collected from each branch to obtain two paired samples of about 20 leaves each. A first sample, to be used for nutrient determination, was stored in a cooler bag during fieldwork, then kept at 4 °C in the laboratory until analysis. The second sample for photosynthetic pigments was immediately packed in aluminum foil, frozen in liquid N, and kept at -80 °C until analysis.

Leaves were washed, oven-dried, weighed, ground and analyzed for C, N, P, K, Ca, Mg, Mn, Fe, Zn, Cu, and B concentrations. The percentage of foliar N was measured with a C/N elemental analyzer (Model NA 1500, Carlo Erba, Milan, Italy). The other nutrients were determined by an Ion Coupled Plasma Atomic Emission Spectrometer (Inductively Coupled Plasma-ICP; Ametek Spectro Arcos, Kleve, Germany) on samples mineralized (US EPA Methods 3052; Kingston 1988) in a microwave mineralizer (Ethos TC, Mileston, Bergamo, Italy).

Photosynthetic pigments (chlorophyll *a*, Chl *a*, chlorophyll *b*, Chl *b,* and total carotenoids) were determined on liquid N_2 homogenized leaf samples according to the method of Wellburn (1994), with some modifications. Briefly, 30-50 mg of leaf material were extracted in 80% (w/v) cold acetone, centrifuged at 3000×g for 10 min at 4°C, filtered, if necessary (0.2-µm, Sartorius Stedim Biotech, Göttingen, Germany), and the absorbance of the supernatant was measured at 663.2, 646.8, and 470.0 nm using an UV-vis spectrophotometer (UV-1800 Spectrophotometer, Shimadzu).

2.3.4. Statistical analysis

All data were averaged over each plot to avoid pseudo-replication. Data were analyzed separately for each sampling year (2016 and 2018) in a complete randomized factorial design with two factors: site (2 levels: Cansiglio and Collelongo) and N treatment (3 levels: Control, Medium and High). For each combination of treatments, three replicate plots (n = 3) were considered. When the two-way analysis of variance (2-way-ANOVA) showed a significant interaction between factors ($p \leq 0.05$), *a posteriori* separation of means was performed by the Student Newman-Keuls test (SNK). An analysis of covariance was also performed to highlight any differences in the relationship between leaf chlorophylls and N content in response to N treatments at the two sites for each sampling date, utilizing the leaves collected on tree branches as sampling units (n = 5-10). A multivariate analysis (Canonical Discriminant Analysis, CDA) was also performed to identify the most relevant factors explaining the variance between and within sites. All statistical analyses were carried out with the R open-source software (R Core Team 2017) using the *easyanova*, *agricolae,* and *candisc* libraries.

2.4. Results

In leaves of both Cansiglio and Collelongo beech forests a significant interannual variability in nutrients and photosynthetic pigments concentration, independently of N additions was shown. For this reason, foliar data from 2016 and 2018 were analyzed separately.

In both sites and years of sampling, most of the macro- and mesonutrients in leaves from the control plots fell within the optimal range suggested by Mellert and Göttlein (2012) and Stefan et al. (1997). In both 2016 and 2018, the leaf N concentration differed significantly between sites, with higher values in Collelongo than Cansiglio, but was not affected by fertilization (Table 2). On the contrary, the leaf P concentration was affected by N additions in 2016, showing a slight decrease in both beech forests with increasing N addition levels: on average more than 4% and 9% at Medium and High N treatment, respectively (Table 2). Remarkably different P concentrations were measured at both sites in the two years, with values consistently above the optimum range in 2016, but close to its minimum value in 2018. In 2016 the leaf K concentration was influenced by both site and N treatment, with generally higher values at Collelongo than Cansiglio and a slight but significant decrease ($p = 0.045$) in response to the higher N dose. In 2018, on the contrary, neither the site nor N addition had effects on the leaf K concentration (Table 2). The Ca concentration was consistently higher in Collelongo than in Cansiglio, in particular in the 2016 growth season ($p < 0.001$), while the Mg concentration was not significantly affected by site, N fertilization or their interaction in either year of sampling (Table 2). Finally, the foliar S concentration was consistently lower in Cansiglio than in Collelongo (-12.5% and -10.3% in 2016 and 2018, respectively) and was significantly affected by N treatment in 2016 only (Table 2). In this year, the N fertilization caused a progressive S reduction of -8.5 % and -16.41 % with the Medium and High N fertilization treatments, respectively, compared to control (Table 2).

Concerning the leaf concentration of micronutrients (Table 3), only Mn and B consistently differed between sites in both growing seasons, being Mn more elevated at Cansiglio than Collelongo, while B higher at Collelongo than Cansiglio. Differences in Fe, Zn, and Cu concentrations were only observed in either one of the two campaigns (Table 3). An effect of fertilization was only observed in 2016, when foliar Fe and Zn concentrations at both sites showed a significant and progressive decline with increasing N doses, which did not persist, however, in 2018. Furthermore, we detected significant increases in Mn concentration in 2018 (effect only in Cansiglio site, 1-way-ANOVA).

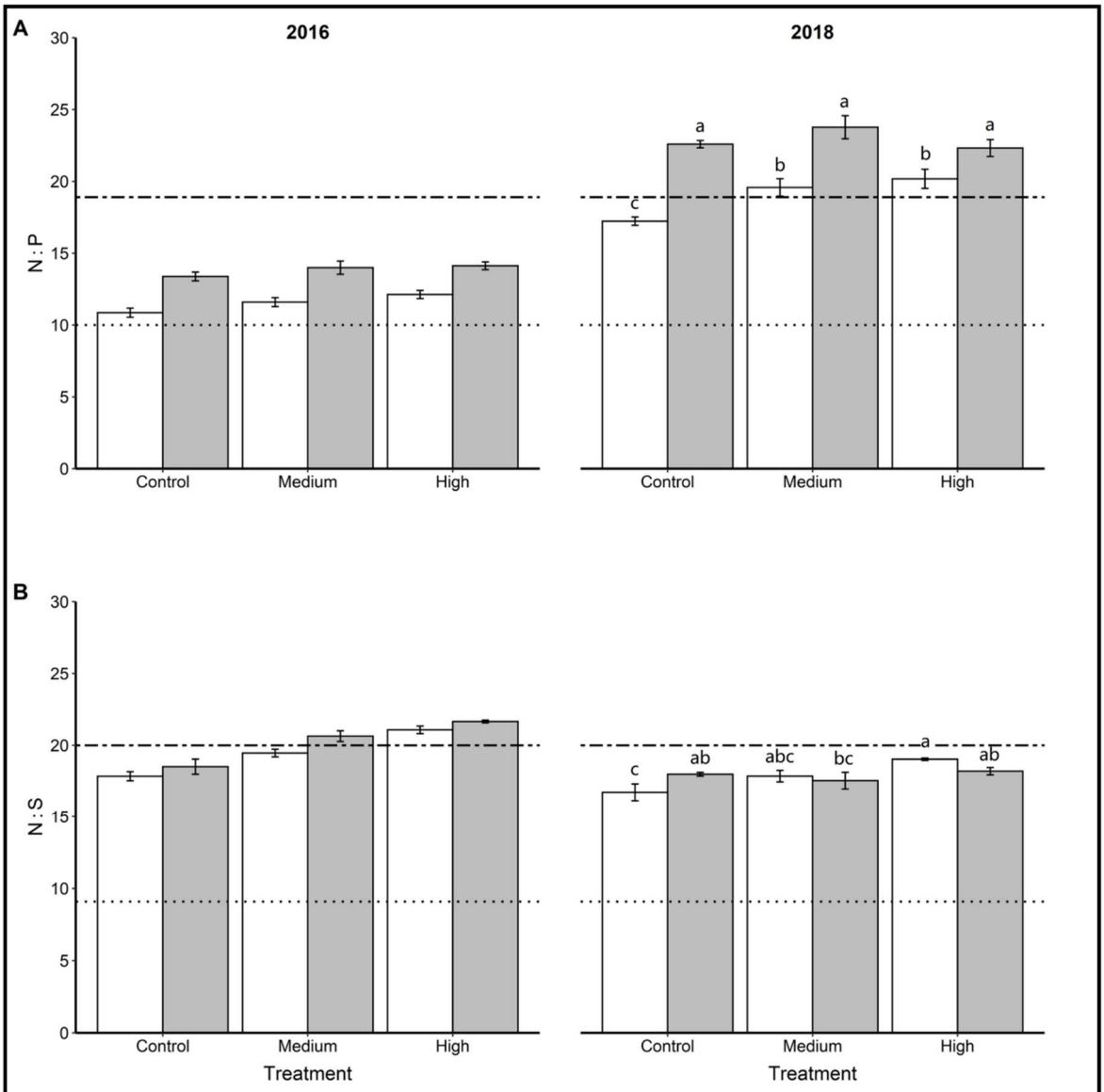
Table 2. Macro- (N, P, K) and meso-(S, Mg, Ca) nutrients concentration in beech leaves collected at the two experimental sites in 2016 and 2018. Mean ± standard error (n = 3). For each parameter, the results of 2-way ANOVA analysis (factors: Site and N treatment) are also presented for each sampling year (2016 and 2018). Values of significant level (p) and F are reported; significant differences ($p \leq 0.05$) are indicated in bold. Fertilization treatments correspond to 0 (Control), 30 (Medium) and 60 kg N ha-1 y-1 (High).

Macronutrients (mg g^{-1})	N				P				K			
Year	2016		2018		2016		2018		2016		2018	
CANSIGLIO												
Control	23.5 (±0.98)		22.0 (±0.61)		2.17 (±0.06)		1.28 (±0.03)		6.05 (±0.21)		5.21 (±0.40)	
Medium	23.6 (±1.04)		22.7 (±1.08)		2.04 (±0.01)b		1.16 (±0.05)		5.76 (±0.25)		5.00 (±0.30)	
High	22.8 (±0.62)		23.6 (±0.35)		1.91 (±0.05)		1.17 (±0.06)		5.51 (±0.29)		4.90 (±0.26)	
COLLELONGO												
Control	27.1 (±0.84)		24.6 (±0.73)		2.02 (±0.06)		1.09 (±0.02)		7.19 (±0.26)		5.53 (±0.48)	
Medium	27.5 (±0.59)		25.6 (±1.39)		1.97 (±0.02)		1.08 (±0.05)		7.75 (±0.09)		5.66 (±0.40)	
High	27.0 (±0.38)		25.0 (±0.58)		1.91 (±0.05)		1.12 (±0.04)		6.60 (±0.37)		5.78 (±0.51)	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Site	37.41	**<0.001**	10.59	**0.007**	1.321	0.273	9.390	**0.010**	44.01	**<0.001**	3.598	0.082
N	0.393	0.683	0.729	0.502	4.647	**0.032**	1.145	0.351	4.070	**0.045**	0.006	0.994
Site x N	0.102	0.904	0.439	0.655	0.933	0.420	1.318	0.304	1.888	0.194	0.261	0.775

Mesonutrients (mg g^{-1})	Ca				Mg				S			
Year	2016		2018		2016		2018		2016		2018	
CANSIGLIO												
Control	9.70 (±0.27)		8.22 (±0.02)		1.75 (±0.12)		1.61 (±0.03)		1.32 (±0.06)		1.32 (±0.02)	
Medium	9.03 (±0.27)		7.93 (±0.42)		1.46 (±0.06)		1.39 (±0.04)		1.21 (±0.04)		1.27 (±0.03)	
High	8.16 (±0.16)		8.71 (±1.00)		1.35 (0.10)		1.53 (±0.10)		1.08 (±0.02)		1.24 (±0.02)	
COLLELONGO												
Control	14.25 (±0.58)		9.55 (±0.48)		1.69 (±0.18)		1.45 (±0.04)		1.46 (±0.04)		1.37 (±0.05)	
Medium	13.70 (±1.93)		10.1 (±0.42)		1.35 (±0.07)		1.42 (±0.15)		1.33 (±0.03)		1.46 (±0.08)	
High	12.22 (±1.00)		9.11 (±1.05)		1.53 (±0.29)		1.42 (±0.14)		1.25 (±0.02)		1.37 (±0.05)	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Site	33.746	**<0.001**	5.658	**0.035**	0.002	0.967	1.068	0.322	19.373	**<0.001**	10.570	**0.007**
N	1.887	0.194	0.019	0.981	2.351	0.138	0.804	0.470	16.395	**<0.001**	0.777	0.482
Site x N	0.060	0.942	0.870	0.444	0.472	0.635	0.465	0.639	0.171	0.845	1.054	0.379

Table 3. Micro-nutrients (Mn, Fe, Zn, Cu and B) concentrations in beech leaves as a function of N fertilization at the two experimental sites (Collelongo and Cansiglio) in 2016 and 2018. Mean ± standard error (*n* = 3). For each parameter, the results of a 2-way ANOVA analysis (factors: Site and N treatment) are also presented for each sampling year (2016 and 2018); significant differences (p ≤ 0.05) are indicated in bold. Abbreviation as indicated in Table 2.

Micronutrients (µg g^{-1})	Mn				Fe				Zn				Cu				B			
Year	2016		2018		2016		2018		2016		2018		2016		2018		2016		2018	
CANSIGLIO																				
Control	0.85 (±0.05)		0.74 (±0.07)		109.7(±13.8)		80.7 (±3.17)		24.2 (±1.26)		24.7 (±0.53)		9.62 (±0.76)		8.71 (±0.36)		34.3 (±0.85)		11.4 (±0.36)	
Medium	1.01 (±0.09)		1.09 (±0.12)		95.4 (±3.39)		86.4 (±4.23)		23.5 (±0.40)		25.9 (±0.49)		9.93 (±0.66)		8.28 (±0.35)		30.3 (±0.39)		10.2 (±0.03)	
High	0.98 (±0.12)		0.95 (±0.08)		89.1 (±3.23)		85.7 (±2.93)		20.1 (±0.83)		24.8 (±2.37)		11.2 (±0.18)		8.54 (±0.37)		28.8 (±0.21)		9.96 (± 0.52)	
COLLELONGO																				
Control	0.85 (±0.06)		0.17 (±0.04)		118.6 (±8.5)		101.1 (±5.6)		18.7 (±0.85)		25.8 (±1.73)		10.7 (±0.35)		7.42 (±0.48)		49.8 (±0.73)		18.5 (±1.76)	
Medium	0.20 (±0.01)		0.15 (±0.01)		97.6 (±2.25)		93.2 (±7.75)		16.9 (±2.65)		25.9 (±3.73)		10.5 (±0.33)		7.13 (±0.47)		48.3 (±3.80)		19.8 (±1.38)	
High	0.33 (±0.04)		0.23 (±0.02)		82.3 (±2.93)		91.9 (±4.09)		14.0 (±1.41)		26.8 (±2.85)		10.4 (±1.46)		7.05 (±0.21)		48.7 (±1.09)		19.5 (±0.83)	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Site	139.0	**<0.001**	164.2	**<0.001**	0.06	0.808	7.67	**0.017**	27.31	**<0.001**	0.31	0.586	0.20	0.661	17.46	**0.001**	166.87	**<0.001**	111.89	**<0.001**
N	0.76	0.487	2.47	0.126	8.28	**0.005**	0.09	0.913	5.15	**0.024**	0.05	0.956	0.41	0.671	0.47	0.636	2.25	0.149	0.05	0.951
Site x N	1.47	0.269	2.32	0.141	0.63	0.549	1.32	0.303	0.07	0.930	0.09	0.915	0.87	0.442	0.10	0.905	0.87	0.445	1.02	0.390



C

Parameter	N:P				N:S			
Years	2016		2018		2016		2018	
Factors	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Site	73.54	**<0.001**	69.37	**<0.001**	8.805	**0.012**	**0.015**	0.906
N	4.836	**0.029**	5.200	**0.024**	46.24	**<0.001**	5.492	**0.020**
Site x N	0.344	0.715	4.068	**0.045**	0.462	0.641	3.855	**0.051**

Figure 1: Leaf N:P and N:S ratios in beech forests at two experimental sites (Cansiglio, white bars; Collelongo, grey bars) subjected to N addition treatments (Control; Medium, 30 N kg ha-1 y-1; High, 60 kg N ha-1 y-1) in 2016 and 2018 (panels A and B). Mean ± standard error (n = 3). Horizontal lines refer to minimum (dotted) and maximum (dashed line) critical values for the species, according to the normal range for nutrient concentrations suggested by Mellert and Göttlein (2012) for N:P ratio and by Stefan et al. (1997) for N:S ratio. Results of two-way ANOVA analysis (factors: site and N treatment, N) are given in panel C. Significant ($p \leq 0.05$) effects of site, N and their interaction are in bold. The columns identified by different letters are significantly different (SNK test).

The N treatments investigated significantly affected the foliar N to P and S ratio in both years of sampling (Fig. 1). During 2016, at both sites, N addition led to a general increase of the leaf N:P

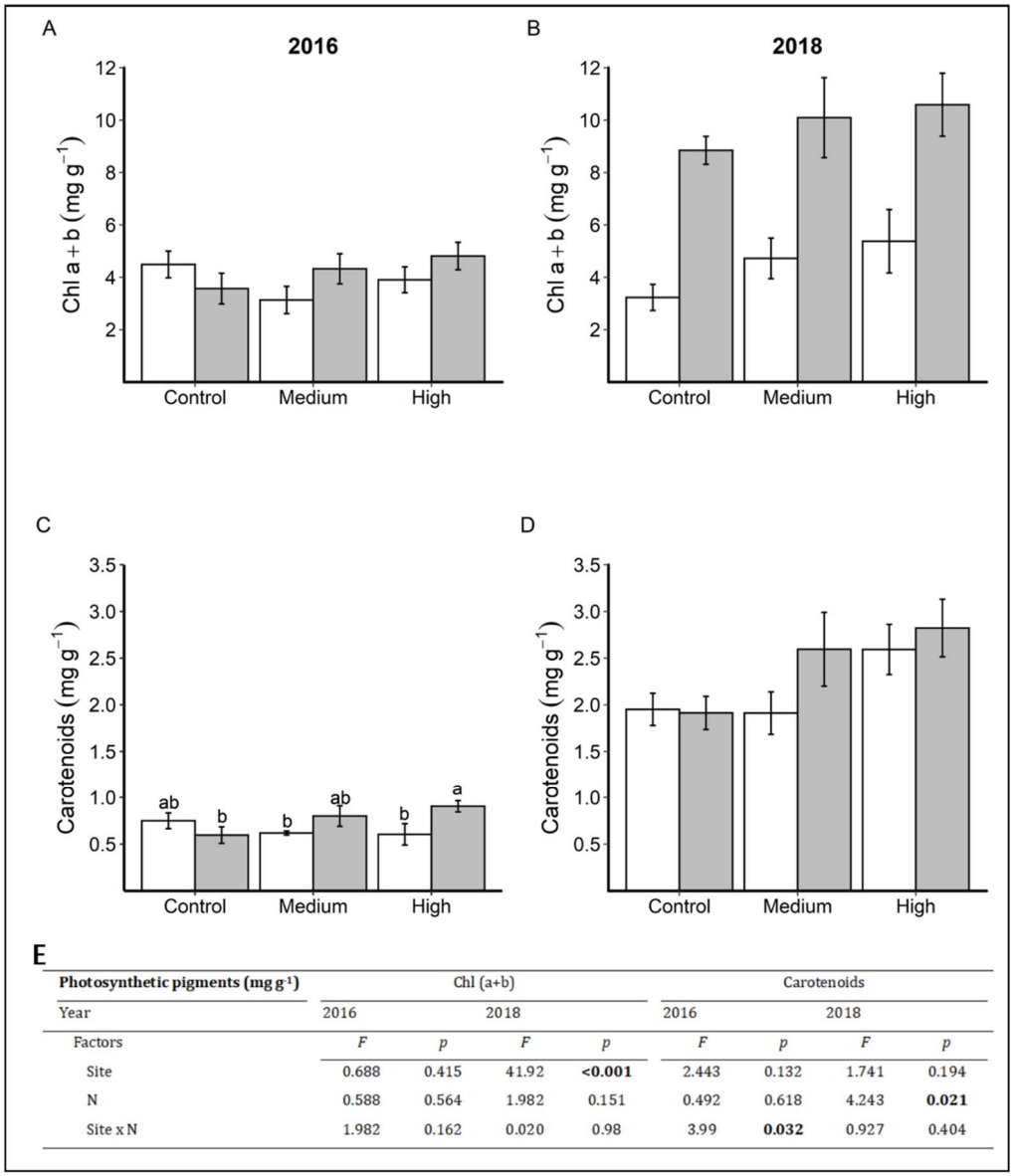
ratio of +6.0 and +8.7 % for Medium and High N treatments, respectively (Fig. 1A, B). In 2018, on the contrary, the response to fertilization differed between sites, with a marked increase in Cansiglio (+14.3% and +16.5% for Medium and High N treatments, respectively) but rather stable N:P values in Collelongo (Fig. 1B, C). In both years, N:P ratios were consistently higher in Collelongo than Cansiglio.

The N:S ratio was also influenced in both sites and study years by N fertilization. While a similar increase was observed at both sites in 2016, in 2018, N addition resulted in a 14% increase in High N treated plots at the Cansiglio site while the increase at Colleongo was not statistically significant (Fig. 1B and Fig. 1C). N treatments did not significantly affect the N to Ca, K, and Mg ratios in either site or year (Table S2), except for N:Fe ratio, which increased significantly in treated plots during the first year of sampling (p=0.006), mostly due to a significant decrease in foliar Fe concentration (p=0.005, Table 3).

In order to better understand the functional significance of the observed changes in leaf nutrients, these have been complemented by paired measurements of leaf pigment concentration. The content of photosynthetic pigments in beech leaves was generally higher in 2018 than in 2016 (Fig. 2 A, B). In year 2016, Chl (*a*+*b*) was influenced neither by site nor by N application or their interaction (Fig.2 E). This was largely due to a relative decrease of both Chl *a* and Chl *b* in response to Medium and High N fertilization levels at Cansiglio, and to a parallel increase at the Collelongo site (1-way ANOVA within site, data not shown), although the interaction between site and N addition was not statistically significant. The Chl *a*/*b* ratio, on the contrary, was significantly affected in 2016 by both site and treatment, but not by their interaction (Table S3). In 2018, Chl (*a*+*b*) was significantly different between sites, with a higher Chl *a*/*b* ratio in Collelongo than Cansiglio (Fig. 2 B, E and Table S3). In both sites, chlorophylls tended to increase with the addition of N: at Cansiglio, Chl (*a*+*b*) progressively increased with N fertilization (1.5-fold with Medium N and 1.7-fold with High N); in Collelongo Chl (*a*+*b*) increased about 1.2-fold with both Medium and High N fertilization (one-way ANOVA within site).

In the 2016 growth season, the leaf carotenoid content of the two beech forests showed a contrasting effect of N fertilization at the two sites (Fig. 2 E), with a decrease at Cansiglio in response to both Medium and High N fertilization (~ -18%) and, on the contrary, an increase at Collelongo in response to the High N fertilization level (+52%, Fig. 2C). In 2018, on the contrary, total carotenoids were positively affected by N at both forest sites (Fig. 2 E), increasing with the Medium N dose at Collelongo (+36%) and with the High N dose at both Cansiglio (+33%) and

Collelongo sites (+48%, Fig. 2 D). The ratio of total chlorophyll to carotenoids (Chls/Car ratio), however, was not affected by fertilization either in 2016 nor in 2018 (Table S3).



E

Photosynthetic pigments (mg g^{-1})	Chl (a+b)				Carotenoids			
Year	2016		2018		2016		2018	
Factors	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Site	0.688	0.415	41.92	**<0.001**	2.443	0.132	1.741	0.194
N	0.588	0.564	1.982	0.151	0.492	0.618	4.243	**0.021**
Site x N	1.982	0.162	0.020	0.98	3.99	**0.032**	0.927	0.404

Figure 2. Total leaf chlorophyll (Chl a+b) and carotenoid concentrations in F. sylvatica trees subjected to N addition treatments (Control; Medium, 30 N kg ha-1 y-1; High, 60 kg N ha-1 y-1) at the Cansiglio (white bars) and Collelongo (grey bars) sites for 2016 and 2018 sampling years (panels A to D). Mean ± standard error (n = 3). statistics as in Fig. 1. Results of the two-way ANOVA analysis (factors: sites and N treatment, N) are given in panel E.

The correlation between leaf N content and Chl (*a*+*b*) was weak and negative at the Cansiglio site in 2016 (r = 0.34, p = 0.014) while the two variables were positively related in 2018 (r = 0.65; p < 0.001, Fig. 3A, B). The relationship was positive and significant at Collelongo in both 2016 (r = 0.64; p < 0.001) and 2018 (R = 0.63; p < 0.001, Fig. 3C, D).

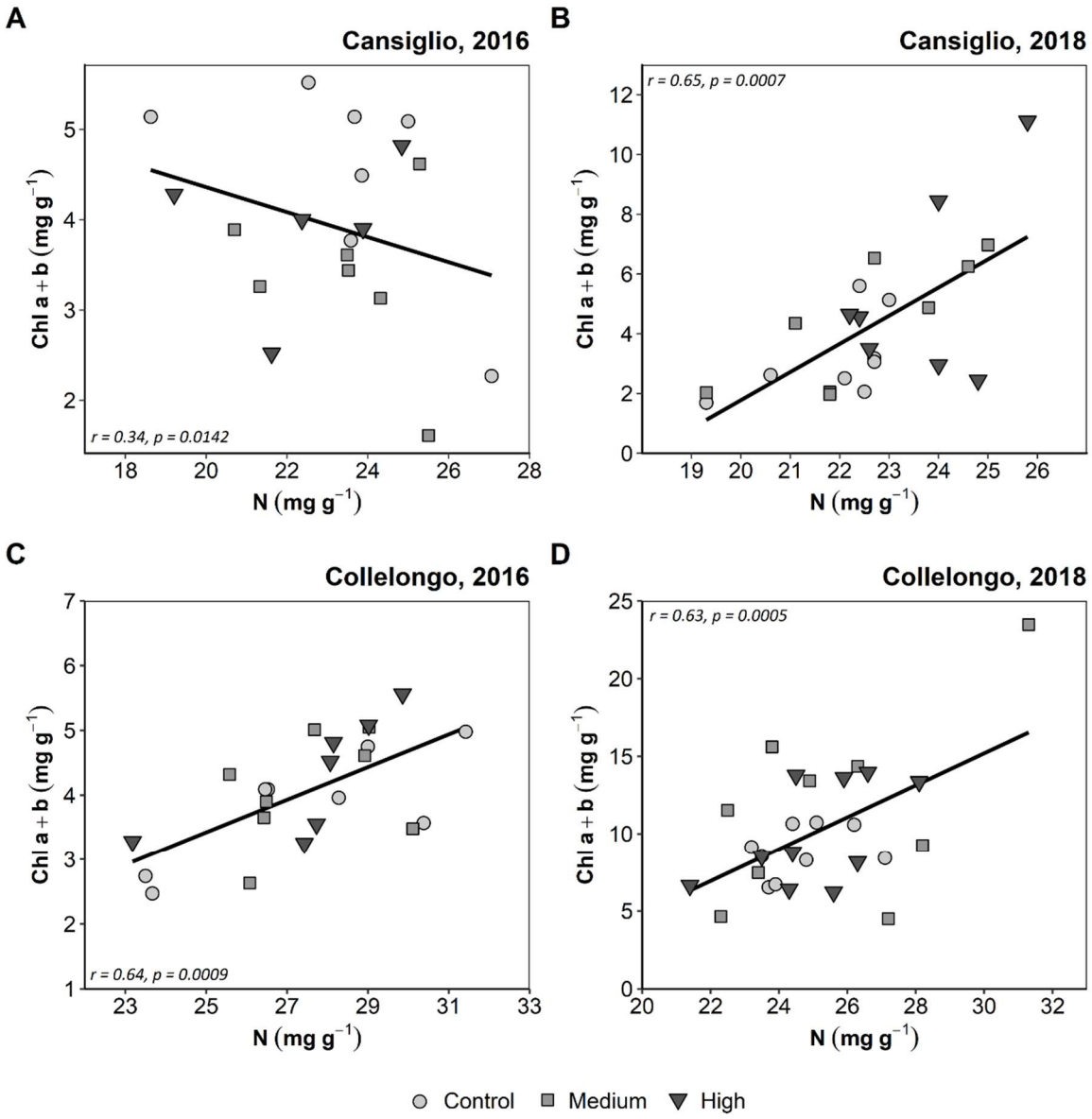


Figure 3. Correlation between leaf N concentration (N) and total chlorophyll concentration (Chl a+b) in Cansiglio and Collelongo forests in 2016 and 2018. Points correspond to measurements on individual plants (n=3-9) from each experimental plot (Control, grey dots; Medium, dark grey squares; High, black triangle during the two sampling campaigns. The Pearson's coefficient and significance level (p) of linear regressions are also shown.

The results of the canonical discriminant analysis (CDA), performed for the foliar nutrient and photosynthetic pigment concentration separately for the two study years (2016 and 2018), are presented in Fig. 4A and 4B, respectively. For each growing season, the CDA finds the canonical coordinates (linear combinations of independent continuous variables, i.e. leaf concentrations of macro-, meso- and micronutrients, chlorophylls, and carotenoids) that best separate the plots belonging to different factor-groups (sites and N treatments). In both years, the first and second canonical variable components accounted for more than 90% of the system's total variation (95.5% and 95.8%, referring to 2016 and 2018, respectively).

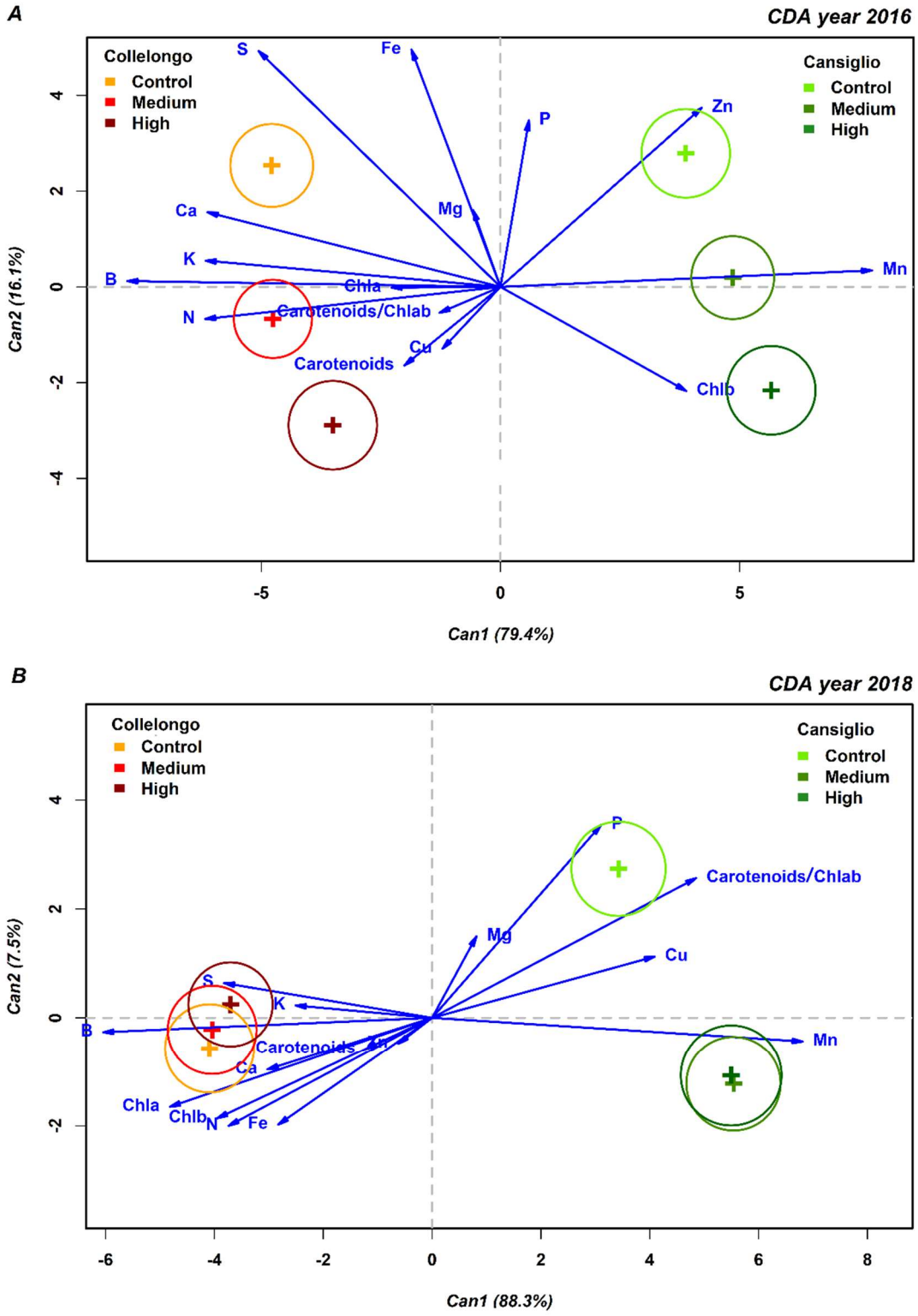


Fig. 4. Principal component analysis showing the difference in nutritional status of F. sylvatica trees at the two sites (Cansiglio and Collelongo) and exposed to different N fertilization treatments (Control, Medium, High), along the first two canonical variate axes during the first (2016, A), and the second year (2018, B) of sampling.

Along the primary gradient of variance (Can1), in both 2016 and 2018, there was an evident separation of data between the sites (Cansiglio and Collelongo), while N treatments within the site were poorly discriminated. The first canonical coordinate, however, markedly differed between the

two years (as apparent from an analysis of the blue arrows in Fig. 4). The N fertilization treatments, on the contrary, were better discriminated along the second gradient of variance (Can2), which explains however less than 20 % of result variation in both years. In the first study year, the second canonical coordinate determined a clear separation among N treatment groups (C, Medium, and High), with the same sequence at both sites (Fig. 4A). However, this second gradient of variance (Can2) explained only 16.1 % of the overall system variability. During the 2018 growing season, on the contrary, the discrimination of N treatments by foliar nutritional status was not confirmed at the Collelongo site, for which all different groups of N fertilization appeared clustered together (Fig. 4B). On the other hand, in Cansiglio, the discrimination of N fertilization treatments observed in 2016 persisted in 2018, but the Medium and High N treatments clustered together and separated from the control plots. Also, in this case, the factors contributing to the second canonical coordinate differed between 2016 and 2018.

2.5. Discussion

Last decades' reports on N deposition in forest ecosystems describe different and sometimes contrasting effects, from the marked increase in biomass production to the lack of growth stimulation, depending on simulated or natural N deposition, timing and mode of exposure, canopy structure, etc. (de Schrijver et al., 2011; Liu et al., 2020; Reay et al., 2008; Y. Zhang et al., 2017). After four years of N treatment, neither a significant increase in radial growth nor in leaf production of the two mature beech stands in northern (Cansiglio) and central (Collelongo) Italy was detected (data not shown). In both forest sites, the main leaf morphological traits (e. g.: Leaf Mass per Area, area, data not shown) were not affected by the N treatments but differed constitutively at the two sites. For these reasons, we decided to refer to all the measurements on a dry weight basis, although no substantial differences were observed on an area basis. Moreover, we chose two Mediterranean forests of similar composition (pure old-growth beech forest stands without understory species) to compare better the effects of N fertilization in two different sites. Nevertheless, apart from the different soil and climatic characteristics of the two forest sites (Fig. S1, Table 1, and Table S1), each habitat presents environmental conditions changing throughout the day and seasons, with a combination of independent parameters that differ in intensity during the year (Pierce et al., 2005; Rezaie et al., 2018). In particular, during spring 2016 Collelongo site was invested by an exceptional late frost, causing complete defoliation, which altered some trees' phenological traits and non-structural carbohydrate dynamics (D'Andrea et al., 2019).

The effects of anthropogenic N deposition on terrestrial ecosystems include changes in growth, nutrient composition, and C and N fluxes (Braun et al., 2020; Eichhorn et al., 2001; Etzold et al., 2020; Zak et al., 2017). In forest ecosystems, where the primary resources are light and soil nutrients availability, in addition to atmospheric element deposition, leaf physiological traits such as the content of nutrients and photosynthetic pigments directly reflect the plant capacity of using these resources.

Cansiglio and Collelongo forests showed a good nutritional status at the leaf level in the plant green-up phase chosen for sampling (July/August); this condition was found to be adequate and indicative of a good nutrient supply in the experimental stands, as indicated by foliar nutritional thresholds (Mellert and Göttlein, 2012; Stefan et al., 1997). In this context, the foliar N concentration at Collelongo site was above the adequate range for beech leaves in both years of sampling, while at Cansiglio the foliar N approached the upper reference limit. Both stands in the two years investigated showed foliar N levels typical of forest ecosystems close to N saturation despite the different N deposition backgrounds. If, on the one hand, N is likely not a limiting

element for the primary production of trees in both sites (Ferretti et al., 2014), on the other hand, it was observed that in the short period, additional inputs of N did not induce severe nutritional deficiency at leaf level. Moreover, although the past coppice management of Collelongo, this site did not appear to be depleted in nutrients; instead, it showed an average higher foliar nutrients concentration than Cansiglio in both years of sampling. Since the Collelongo site showed an average higher soil cation exchange capacity and base saturation than Cansiglio (Table S1), foliar nutrient composition confirmed that foliar stoichiometry could be a valuable tool to assess the supply of nutrients in the forest soil (Cleveland et al., 2011). In 2018, in both stands, fruit production was significantly higher than in 2016. Elevated fruit production is thought to be a high energy-demanding process for trees, leading to a consistent depletion of individuals' stored resources due to substantial investment in sexual reproduction (Fernández-Martínez et al., 2017; Sala et al., 2012; Yasumura et al., 2006). This, with high probability, had consequences on the foliar nutrient's stoichiometry (Güsewell, 2004), which resulted in a consistent reduction in the majority of foliar macro and meso-nutrients (especially P, K, and Ca) and significant higher N:P ratios compared to the 2016 growing season. Leaf P concentrations measured agree with other monitoring and manipulative studies (Braun et al., 2010; Gradowski and Thomas, 2008, 2006; Prietzel and Stetter, 2010), observing that soil acidification due to N deposition and climate change negatively affect P availability in forest ecosystems. The low P availability, its decrease in leaves, or a high N:P ratio were also found in Swiss (Braun et al., 2010), French, Belgian, and Luxemburg beech forests (Jonard et al., 2010) under N deposition. The foliar P concentrations significantly decreased with increasing doses of N applied in 2016, when the P availability for plants was more elevated, given the high foliar content of this nutrient even in control plots of both sites (Table 2). The depletion of foliar P with high N addition was also found in one experiment with maple trees treated with chronic N additions at the Harvard Forest (Minocha et al., 2000). In 2018, the foliar P concentration was lower than in 2016, regardless of the N treatments, and was not significantly influenced by the N supply. This might be explained by the *F. sylvatica* attempt to maintain a certain degree of leaf homeostasis, trying to preserve the foliar nutrients concentrations within the optimum range. This mechanism might allow the plant to maintain the leaf physiological functions, compensating for the limited availability of nutrients in the soil by re-mobilizing the stored P in the reserve organs, such as the root system (Zavišić and Polle, 2018). If this hypothesis was true, the general decrease in the P foliar concentration in European forests could be both a symptom of the decline in soil P availability and a consequent depletion of this nutrient content in the plants' reserve organs.

Foliar S concentrations during 2016 decreased significantly in the plots treated with N fertilization. Chen et al. (2018) observed that N addition had inhibitory effects on the activity of soil arysulfatase (an enzyme involved in the mineralization of organic S) and suggested that N deposition may decelerate soil S cycling. At Collelongo, the leaf S reduction could also be explained by the physiological unbalances that occurred to the canopy after the foliar depletion and the reserve remobilization caused by an exceptional late frost (D'Andrea et al., 2021, 2019).

The increase of N:P and N:S ratios in both sites and years of samplings can be partially related to the relative increase of the foliar N concentration (although not significant in any experimental condition), and partially associated with the reduction of foliar P and S content, which showed a decreasing trend in both sampling years, significant for 2016. As well as N:P and N:S ratios, also N:Fe ratio was significantly altered by N fertilization in 2016. A pot experiment on N fertilization of young beech plants (Flückiger and Braun, 1998) caused an increase in N/other nutrients ratios; however, in that experiment, a considerable amount of N (200 kg ha^{-1} yr^{-1}) was applied, which hardly reflects the actual effect of N deposition in forest ecosystems, contrary to the aim of our research. In our case, the observed leaf N:P and N:S variations could indicate that the beech nutrient demand can be almost totally supplied by the soil nutrient pool available in both experimental sites and that N deposition mainly influences the S and P availability (Berger et al., 2016). For what concerns the N:Fe ratio, a similar response was also found by Liu et al. (2021) in grassland ecosystems after three years of chronic N additions. The addition of N, could result in a reduction of soil pH, which involves an enhanced Fe and Mn mobility (Haynes and Swift, 1985; Thomson et al., 1993). Despite this, we found a significant decrease in foliar Fe in 2016, while we detected increases in Mn concentration in 2018 in Cansiglio site. Increased foliar Mn concentrations were also found by Flückiger and Braun (1998) in trees subjected to high N deposition. Based on this evidence, it is possible that the increased Mn mobility in the soil has an inhibitory effect on plants' Fe uptake, as already observed by other authors (Cai et al., 2017; Kobayashi et al., 2019). The concentration of the other foliar micronutrients did not vary in function of N treatments, except for Zn foliar concentration, which showed a significant reduction in treated plots only in 2016. The micronutrients Mn, Fe, Zn, and Cu are redox-active metals involved in the photosynthetic processes, and Fe is also fundamental in chlorophylls biosynthesis (Schmidt et al., 2020; Shahid et al., 2016). In any case, Fe concentration was more consistent in the beech leaves of Collelongo than Cansiglio, reflecting the relatively higher content of Chl a and Chl b in this site. On the contrary, most of the other microelements seem to be more available in Cansiglio, probably due to the higher soil acidification (Table S1).

The increase of leaf chlorophyll content or photosynthetic rate is usually positively correlated with high N availability, although it is strictly dependent on the kind and physiological status of forests (species, age, and nutrient condition) and climatic conditions (Liu et al., 2019; Tavarini et al., 2016; Yan et al., 2013). In our study, leaf N content was higher in Collelongo beech forest than Cansiglio, independently of N distribution, as well as Chl (*a*+*b*), especially in the 2018 growing season. These results are consistent with the positive and significant correlation found between foliar N concentration and Chl (*a*+*b*), except at the Cansiglio site in the first year of sampling (Fig. 3A). The late frost event of 2016, which provoked remobilization of C reserves and a re-growth of the canopy after defoliation at Collengo (D'Andrea et al., 2021), probably impaired the N assimilation and allocation between the photosynthetic apparatus (chlorophylls and proteins) and other metabolic pathways. Nevertheless, a similar unchanged or reduced value of Chl (a+b) was observed in other experiments of N deposition (Arróniz-Crespo et al., 2008; M. Li et al., 2018). The relatively slow response of beech forest at Cansiglio site to three years-N fertilization with regard to the leaf concentration of chlorophylls might be related to the different climatic conditions and plant stand characteristics (density, age, canopy architecture, management, etc.) of the two sites (R. Li et al., 2018; Phoenix et al., 2012; R. Zhang et al., 2017). However, if in some cases total leaf chlorophylls content cannot be an appropriate indicator of N deposition effects, further measurements of photosynthetic pigment compositions such as Chl *a*/*b* and carotenoids are suggested to comprehensively reveal the impacts of N addition on photosynthesis-related parameters and plant biomass performances (e.g., Arróniz-Crespo et al., 2008; M. Li et al., 2018; Ochoa-Hueso et al., 2014; Zhang et al., 2016). In both northern and central beech forest, Chl *a*/*b* was reduced by N fertilization in the 2016 growing season, due to a decrease of Chl *a* at Cansiglio and a relatively higher increase of Chl *b* than Chl *a* at Collelongo site. Similarly, in *Leymus chinensis* Zhang et al. (2016) found a significant rise in Chl *b* due to N addition, while M. Li et al. (2018) revealed unchangeable or enhanced values of both Chl *a* and Chl *b* in *Cupressus lusitanica* after different N treatments. In both beech forest sites, the leaves sampled are typical sun leaves developed in full light, with relative elevated Chl *a*/*b* ratio (> 2.7-2.8), mainly due to a minor investment of Chl *b* in PSII (Scartazza et al., 2016). The Chl *a*/*b* ratio is a valuable indicator of N partitioning within a leaf because it is positively correlated with the ratio of PSII cores to light harvesting chlorophyll-protein complex (LHCII) (Terashima and Hikosaka, 1995). It has been predicted that the ratio of PSII to LHCII (and the Chl *a*/*b* ratio) should increase with the decreasing of N availability (Kitajima and Hogan, 2003), while it has not been demonstrated the contrary, that is the increase of N availability for plants should be associated with a decrease of leaf Chl *a*/*b* ratio, as in our case. However, N

plays important roles in photosynthesis and the production of light-harvesting pigments and diphosphoribulose carboxylase (Wu et al., 2008), and variations in leaf Chl *a*/*b* are usually related to morphophysiological/structural differences in plants and/or to plant response to alterations involving re-arrangements and distribution of photosynthetic pigments between the two photosystems (Maina and Wang, 2015; Y. Li et al., 2018). This could explain the constitutive differences found in leaf Chl *a* and Chl *b* concentrations found at Cansiglio and Collelongo, as well as the modification of Chl *a*/*b* and carotenoids during the 2016 and 2018 growing season. Moreover, the relative increases of carotenoids observed at Collelongo site with both N doses during 2016 and 2018, and at Cansiglio with the High N treatment in 2018 (Fig. 2 C, D) can be due to an up-regulation of the synthesis of these pigments in order to protect the photosynthetic apparatus to possible photo-oxidative damages caused by the effects of N depositions, as found in similar experimentations (M. Li et al., 2018; Zhang et al., 2016).

The multivariate analysis helped to identify the most representative factors influencing this complex forest system, highlighting the clear differences between the two forest stands. The site of Cansiglio showed an overall higher response to further N inputs than the site of Collelongo in both years of sampling. It is known that the effects of N addition differed depending on abiotic factors, such as soil pH and exchangeable base cations (Mao et al., 2020). The higher soil pH and cation exchange capacity in Collelongo might represent a better-buffered system against acidification (Bowman et al., 2008) than in Cansiglio. These soil properties may explain why we found fewer imbalances in foliar nutrients stoichiometry for the central site.

In accordance with the result of the CDA, imbalances in nutrients stoichiometry due to N fertilizations were enhanced during the 2016 growing season (Fig. 4 A), when both sites had relavely low fruit production, and Collelongo was interested by a consistent late frost event (D'Andrea et al., 2019; 2021). N additions determined a detectable enrichment of N foliar concentration in the northern site, combined with an overall depletion of all macro and meso-nutrients, except for Ca in 2018 (Fig 4 A-B). In contrast, in the southernmost site, treatments did not significantly affect foliar nutrients concentration in the last sampling year (Fig. 4 B). Hence, the interaction with the late spring freezing event may have emphasized the effect of N fertilization on foliar nutrients concentration at the site, which we did not observe during the 2018 growing season. Although profuse studies had already demonstrated the consistent reduction of beech forest productivity during years interested by frosts events (e.g. D'Andrea et al., 2020; Gazol et al., 2019; Vitasse et al., 2019), much less is known about the impact on canopy physiology and

morphology. Even if Collelongo forest had to mobilize C reserves to produce a second leaf flush after the severe frost foliar damage (D'Andrea et al., 2021), nutrients concentrations did not drop below deficiency thresholds for optimal physiological functions (Mellert and Göttlein, 2012; Stefan et al., 1997). Nevertheless, we observed a consistent reduction of P, S, Ca, and K foliar concentration in High N fertilized plots compared to control plots, which we did not find during the 2018 growing season. St. Clair et al. (2009) observed a similar reduction in foliar nutrients concentration after a defoliation event in Aspen trees, but the study did not consider interaction with N depositions. As other authors suggested, excess of N could directly affect trees' sensitivity to early frost episodes in spring (Jönsson et al., 2001). Hence, an enhanced frequency of climate extreme events could lead to higher depletion of foliar nutrient resources in presence of elevated N deposition, potentially mining the health and resilience of the forest ecosystems.

Photosynthetic pigment concentrations also varied more remarkably in 2016 than in 2018 growing seasons, with impairments in the relative content of Chl *a* to Chl *b* in both sites in the first year, in addition to changes in carotenoids, which persist in 2018. This was also demonstrated by the different directions of Chl *b* in respect to the other pigment components in 2016 and of carotenoids to total Chls ratio in 2018 (Fig. 4). Photosynthetic pigments are early sensitive indicators of the plant response to environmental stresses (Y. Li et al., 2018; Sharma et al., 2019; Zhang et al., 2016), so their changes, more evident in 2016 in both sites, might reflect the level of plant acclimation to the increased N deposition.

2.6. Conclusions

We found that N addition differently affected the physiological performance of two monospecific beech forests, mainly due to differences in climatic conditions and structure and forest management. Nevertheless the two sites are never compromised with evident deficiencies or leaf chlorosis. In the Cansiglio forest of the Northern Italian region, the pattern of nutrients and pigments differed after the first year of N addition (2016), but such effects did not significantly distinguished the plots treated with the addition of Medium and High N from the control. Also, in the Southern beech forest, the response to N treatments was already evident from 2016, but then the environmental conditions seemed to buffered these differences making the treated plots comparable to the control. We suggest that eutrophic beech forests, developed on nutrient-rich soils for several years, do not manifest, at least in the four years monitored, critical nutritional and physiological imbalances in response to N addition, but but modifications indicative of an incipient stress response. In particular, the southern site, characterized by lower N deposition background

and better soil fertility conditions, showed the highest homeostasis in foliar characteristics in response to N addition.

The leaf physiological and stoichiometric response to N addition of the two beech forests was not remarkable, but the modifications observed in nutrients composition and photosynthetic pigments concentration might be indicators of early plant response to an imbalance induced by N deposition. These leaf physiological and nutritional traits have proved to be good indicators of beech acclimation to environmental and seasonal changes due to N deposition, giving pigments an earlier response while nutrients a later and more resilient one, being these elements subjected to the complex relations of soil availability and plant biomass partitioning. All fundamental characteristics for the monitoring of complex ecosystems such as beech forests, whose soil origin and age influence the short and long term response. Indeed, both the sites analysed developed in relative reach and fertile soils, although with substantial differences,. Favorable stationary conditions might likely enhance the resilience to N additions of stands. On the other hand, forest ecosystems developed on poor soil with a low supply of base cations might be more vulnerable to an excess of N deposition experiencing, even in the short term, marked nutritional imbalances, growth disturbance, and increased leaching of nutrients in the soil. Moreover, we suggest that with the occurrence of other environmental stresses (such as drought, late frosts, and heat waves), imbalances induced by N deposition might be further emphasized, reaching critical thresholds.

Finally, further investigations over an extended period are needed to understand if the observed variations in leaf stoichiometry and pigment compositions result from an optimal acclimation of beech or impaired physiology under the tested N additions.

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2.8. Supplementary materials

Table S1. Main properties of the soils at Collelongo (Humic Alisol) and Cansiglio (Haplic Luvisol). Data collected at the permanent sites ABR1 and VEN1, part of the Italian ICP Forests component (CONECOFOR program); CEC: Cation-exchange capacity, BS: Base Saturation.

Site	Depth (cm)	pH in H_2O	C org (%)	N tot (%)	C/N	Exchangeable base cation (cmol kg^{-1})				Exchangeable Acidity (cmol kg^{-1})	CEC (cmol kg^{-1})	BS (%)	Texture		
						Mg	Ca	Na	K				Sand (%)	Silt (%)	Clay (%)
Collelongo	0-10	5.5	13.6	1.01	12.9	2.46	27.5	0.16	1.34	1.45	32.9	95.6	78.6	19.5	1.9
	10-20	6.0	7.97	0.68	11.7	1.35	25.2	0.15	0.85	2.09	29.6	92.9	71.0	24.6	4.4
	20-40	6.4	6.28	0.57	11.0	0.97	23.8	0.14	0.62	1.87	27.4	93.2	65.6	26.2	8.2
	40-80	5.8	2.95	0.31	9.6	1.04	6.4	0.14	0.69	4.26	12.5	66.0	40.5	36.8	22.7
Cansiglio	0-10	4.2	4.14	2.6	1.6	1.0	8.0	0.15	0.72	6.7	16.6	59.7	39.6	51.3	9.1
	10-20	4.7	1.63	0.50	3.4	0.7	8.4	0.14	0.47	5.9	15.6	62.3	30.7	57.4	11.9
	20-40	5.0	1.15	0.27	4.3	1.0	14.9	0.15	0.62	5.4	22.0	75.5	25.0	60.7	14.3
	40-80	5.2	0.74	0.12	6.3	1.4	19.4	0.15	0.90	6.1	28.0	78.2	20.0	60.5	19.6

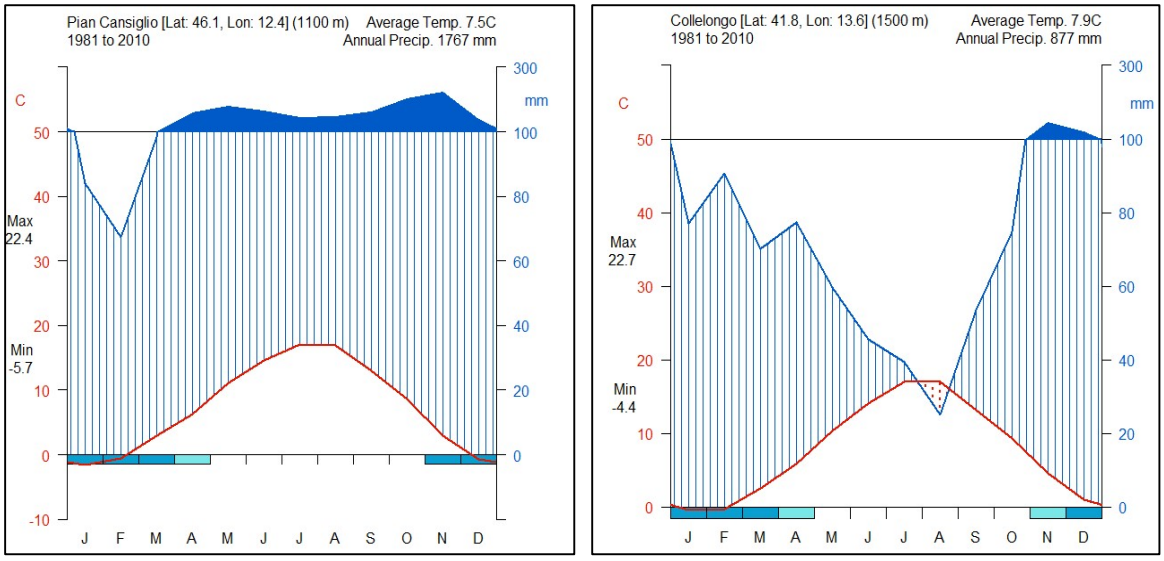

Fig. S1. Walter and Lieth diagrams for the two sites, derived from long term (1981-2010) daily measurements at nearby meteorological stations.

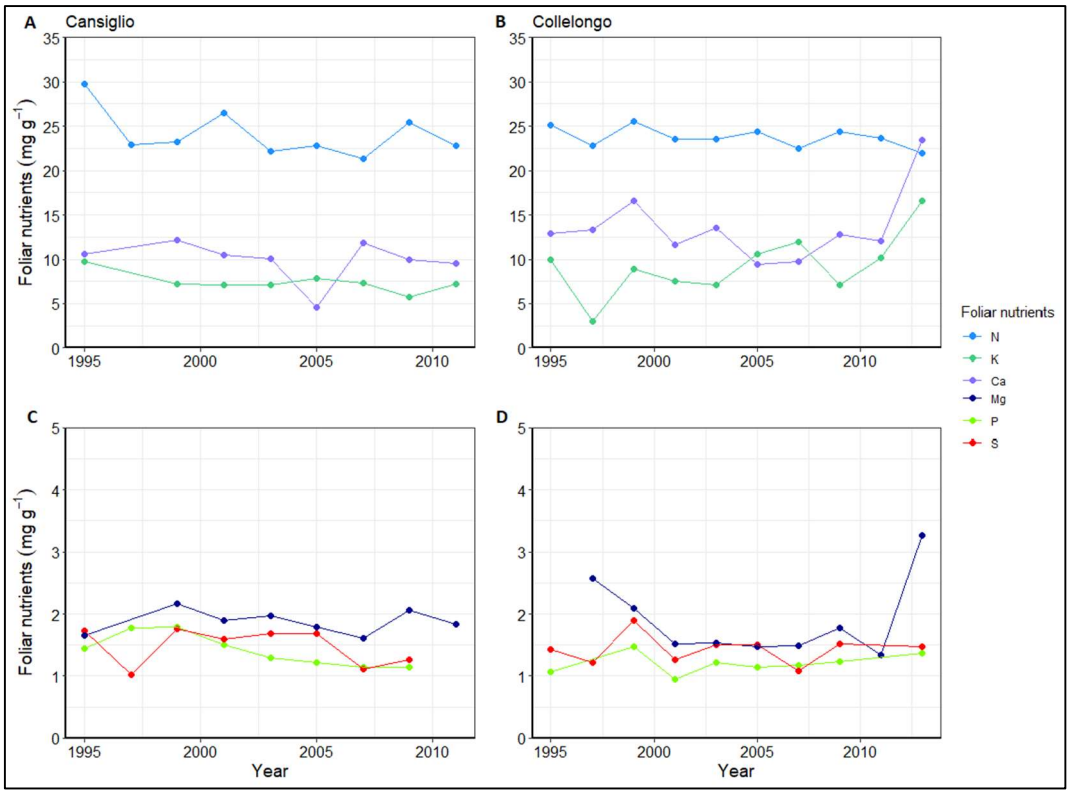

Fig. S2. Historical series (1995-2014) of leaf nutrients concentration in beech forests of ABR1 and VEN1 sites (CONECOFOR program).

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Table S2. Foliar N to K, Ca and Mg nutrient ratios in beech leaves at the two experimental sites. Data refer to mean ± standard error (n = 3). For each parameter, the results of a two way-ANOVA analysis (factors: site and N treatment) are presented for each sampling year (2016 and 2018). Significant effects (P≤ 0.05) are in bold.

	Cansiglio					
Year	2016			2018		
Parameter	N:K	N:Ca	N:Mg	N:K	N:Ca	N:Mg
Control	3.89±0.12	2.43±0.09	13.53±0.41	4.27±0.34	2.67±0.07	13.69±0.24
Medium	4.10±0.10	2.62±0.07	16.14±0.19	4.54±0.64	2.88±0.23	16.31±0.83
High	4.15±0.23	2.79±0.08	16.96±0.93	4.84±0.19	2.77±0.26	15.52±0.79
	Collelongo					
Year	2016			2018		
Parameter	N:K	N:Ca	N:Mg	N:K	N:Ca	N:Mg
Control	3.77±0.06	1.90±0.08	16.32±1.77	4.52±0.37	2.60±0.22	16.99±0.19
Medium	3.55±0.04	2.09±0.30	20.52±1.47	4.53±0.07	2.54±0.15	18.64±2.85
High	4.12±0.28	2.23±0.14	18.70±2.86	4.39±0.43	2.81±0.30	17.96±1.66

Two way ANOVA	*P*	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*	F
Site	0.100	3.181	**0.001**	18.878	**0.038**	5.435	0.764	0.095	0.494	0.499	**0.041**	5.271
N	0.137	2.355	0.113	2.632	0.101	2.788	0.730	0.324	0.789	0.242	0.350	1.146
Site x N	0.274	1.443	0.991	0.009	0.703	0.363	0.486	0.767	0.672	0.411	0.934	0.068

Table S3. Results of the 2-way ANOVA for photosynthetic pigments. The two factors analysed were site (Cansiglio and Collelongo) and nitrogen addition rate (N) with three levels (0, 30 and 60 kg N ha^{-1} y^{-1}) for each year of sampling (2016 and 2018). Chlorophyll *a*, Chl *a*; chlorophyll *b*, Chl *b*; total chlorophylls, Chl*(a+b)*; carotenoids, Car; total chlorophyll to carotenoid ratio, Chl/Car. For each parameter, significant differences ($P \leq 0.05$) are indicated in bold.

Parameter	Factors	F	*p*	Parameter	Factors	F	*p*
Chl *a*, 2016	Site	3.356	0.079	Car, 2016	Site	2.443	0.132
	N	0.474	0.629		N	0.492	0.618
	Site x N	1.824	0.183		Site x N	3.990	**0.032**
Chl *a*, 2018	Site	47.246	**<0.001**	Car, 2018	Site	1.741	0.194
	N	1.469	0.242		N	4.243	**0.021**
	Site x N	0.040	0.961		Site x N	0.927	0.404
Chl *b*, 2016	Site	1.145	0.295	Chl *a*/*b*, 2016	Site	38.452	**<0.001**
	N	1.089	0.352		N	6.543	**0.005**
	Site x N	1.979	0.160		Site x N	2.497	0.103
Chl *b*, 2018	Site	26.079	**<0.001**	Chl *a*/*b*, 2018	Site	18.39	**<0.001**
	N	2.936	0.064		N	0.587	0.560
	Site x N	0.002	0.998		Site x N	0.476	0.625
Chl*(a+b)*, 2016	Site	0.688	0.415	Chl/Car, 2016	Site	1.097	0.305
	N	0.588	0.564		N	1.178	0.325
	Site x N	1.964	0.162		Site x N	1.569	0.229
Chl*(a+b)*, 2018	Site	41.922	**<0.001**	Chl/Car, 2018	Site	33.291	**<0.001**
	N	1.982	0.151		N	0.414	0.664
	Site x N	0.020	0.980		Site x N	2.443	0.099

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3. Forest nitrogen dynamics in response to increasing nitrogen deposition: comparing above-canopy and soil fertilization in a mature beech forest

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3.1. Summary

Elevated atmospheric nitrogen (N) deposition poses a significant threat to forest ecosystem functions, with its effects contingent on the intricate fate of N within the ecosystem and the pivotal role of tree canopies in intercepting, transforming, and assimilating atmospheric N inputs. The present work investigates how N deposition can alter the balance between N retention and loss in the ecosystem. The Cansiglio beech forest, located in the Belluno Prealps, is subject to one of the highest N deposition rates in Italy. On this site a manipulative experiment was established in 2015 to simulate N deposition in the forest. N is distributed in inorganic form (NH_4^+-NO_3^-) three times during the growing season. Treatments are replicated in two different doses: (i) 30 kg N ha^{-1} y^{-1}, distributed both directly to the soil and above the canopy, and (ii) 60 kg N ha^{-1} y^{-1} distributed exclusively to the soil. To characterize the key processes underpinning ecosystem N cycle, we integrated measurements of N concentration and $\delta^{15}N$ in various compartments along the forest profile— including leaves, fine roots, ectomycorrhizal root tips, soil, and decomposed and undecomposed litter. Additionally, we employed molecular analyses to assess potential changes in the functional genes associated with key N processes under elevated N addition. Our results indicate that N additions did not significantly alter potential N losses and retention soil processes, irrespective of the fertilization approaches and N dose applied. Overall, treatments did not significantly alter N concentrations across most of the compartments investigated. However, $\delta^{15}N$ in plant tissues emerged as a more sensitive indicator of N cycling in forest ecosystems, effectively depicting differences among treatments in the alterations of the soil-plant N pathways, which followed different trajectories in relation to the nitrogen addition approaches (canopy vs soil applications). This has important implication on the next-generation of N manipulation experiments, which should more realistically simulate changes in global change drivers, such as N deposition.

3.2. Introduction

Most of the reactive nitrogen (N) input from the atmosphere to terrestrial ecosystems comes from anthropogenic activities, such as chemical fertilizer synthesis, intensive livestock, and fossil fuel combustion (Galloway et al 2004). These processes have significantly altered the N cycle and, consequently, the availability of this nutrient in temperate forest ecosystems, which are commonly N-limited (Galloway et al 2008; Rennenberg and Dannenmann 2015). N deposition can enhance photosynthesis (Janssens and Luyssaert 2009), tree growth (Etzold et al 2020) and forest carbon sequestration (Magnani et al 2007; Wang et al 2017; Schulte-Uebbing and de Vries 2017). However, if in excess, they can induce forest ecosystem saturation, with adverse effects on forest productivity and functionality (Takahashi et al 2020). Indeed, N saturation is a process wherein N input to the ecosystem exceeds its biological demand, fostering N losses through leaching, denitrification, and ammonia volatilization (Aber et al 1989). Thus, the rise in N deposition has the potential to adversely impact the functions of ecosystems. The extent and the timing of these negative effects, however, depends on the status of the ecosystem and its capacity for N retention (Aber, 2002).

Numerous studies demonstrated that increasing atmospheric N input to ecosystems leads to elevated rates of internal N cycling, which can be captured by measuring stable nitrogen isotope ratios ($^{15}N/^{14}N$) in forest samples (Craine et al 2018), expressed as stable isotope composition ($\delta^{15}N$). Key soil microbial processes, including decomposition, mineralization, nitrification, and denitrification, preferentially discriminate against the heavier isotope (^{15}N) (Hobbie and Ouimette 2009; Hogberg 1997; Hobbie and Colpaert et al 2003). Consequently, the products of these processes are relatively lighter in ^{15}N, or less enriched, while the substrate from which they originated becomes heavier, or more enriched in ^{15}N (Peterson and Fry 1987; Yoneyama 1996). Thus, at elevated N cycling rates, soil-available N is more enriched in the heavier isotope ^{15}N, given that the lighter isotope ^{14}N can preferentially exit the ecosystem through processes such as denitrification or NH_3 volatilization (Hogberg 1997). As plants take up the remaining, relatively enriched N in the soil, they also can become relatively enriched in ^{15}N (and hence show more positive $\delta^{15}N$ values) as they reflect the N they assimilate (Templer et al 2007). Indeed, numerous studies have demonstrated a robust positive correlation between foliar $\delta^{15}N$ and soil N concentration and N process rates (Emmett et al 1998; Meints et al 1975; Hogberg, 1990; Gebauer and Schulze 1991, Craine et al 2015). Although the inherent complexity in interpreting data from ^{15}N natural abundances in terrestrial ecosystems, they are commonly employed as tracers or proxies for ecological processes (Craine et al 2015). This approach offers several advantages,

primarily due to the ease of measurement and the relatively lower cost of using natural abundance of N isotopes compared to techniques involving enriched ^{15}N isotope tracers (Templer et al 2007). Over the last few decades, numerous N manipulation experiments have been conducted to understand the impacts of increasing N deposition on forest ecosystems. However, in the "old-generation" fertilization experiments, the applied N doses often greatly exceeded the actual atmospheric deposition (de Schrijver et al 2011; Hyvönen et al 2008; Billow et al 1994; Kaakinen et al 2009). Moreover, the increase in atmospheric N input was typically simulated by direct soil applications, excluding *a priori* potential interactions between atmospheric N and tree canopies. Indeed, there are mounting evidences that tree canopies can intercept up to 70% of atmospheric N deposition (Sievering et al 2007; Gaige et al 2007; Dail et al 2009). While part of the intercepted N may undergo transformation or volatilization, being lost from the ecosystem, a portion of it could be directly assimilated by the leaves, thus being readily available for plant N metabolism (Sparks 2009), or it can be used by microbes hidden in the phyllosphere (Guerrieri et al 2021). Therefore, direct-to-soil N additions might have overlooked important processes and inaccurately simulated the fate of N deposition and its impact on forest biogeochemical processes (Bortolozzi et al 2021; Da Ros et al 2023). Indeed, most of the soil N manipulation experiments reported evidence of N saturation with increasing N doses applied, which was detected by using $\delta^{15}N$ in soil and foliar samples (Nadelhoffer et al 1999; Magill et al 2004). Field studies reported an increase in NO_3^- leaching at forest sites exceeding N depositions of 10 kg ha^{-1} y^{-1} (Gundersen et al 2006), but there is no clear evidence that this pathway of N loss (and hence saturation) increases over time under increasing N deposition. In contrast, recent analyses (including also $\delta^{15}N$ in foliar samples) have identified pervasive N limitations in forests (Craine et al 2018; Mason et al 2002), which has negative implication on the expected CO_2 fertilization effect on carbon sequestration. As the impacts of N deposition on forest ecosystems involve intricate dynamics, it is urgent to investigate how increasing levels of N deposition alter plant-soil interactions, for achieving a more comprehensive understanding of the influence of N deposition on the interplay between N and carbon cycling in these ecosystems.

The aim of this study was to investigate the alterations in the N cycle, with particular reference to retention vs. saturation ecosystem N dynamics. Through the implementation of both direct-to-soil and above-canopy N fertilizations established in a mature deciduous forest, we assessed the unique opportunity to assess whether soil fertilizations overestimate the effects of N deposition on ecosystem N dynamics. We tested the following hypotheses: 1) N addition would increase N availability, regardless of the approach (above-canopy vs. direct-to-soil fertilization) and doses

applied; 2) direct to soil fertilization would accelerate the N cycle, resulting in increased N concentration and $\delta^{15}N$ across various ecosystem compartments; 3) the higher N availability may induce changes in tree N-uptake strategies; finally, 4) canopy N interception would mitigate the impact of simulated increase in N deposition on the forest soil, in the case of canopy application compared to direct-to-soil fertilization. To test our hypotheses, we combined the measure of N concentration and $\delta^{15}N$ in different compartment along the forest profile (i.e., leaves, fine roots, ectomycorrhizal root tips, soil, and decomposed and undecomposed litter) with molecular analyses to evaluate potential changes in the functional genes associated to key N processes under elevated N addition.

3.3. Material and Methods

3.3.1. Experimental site

The study site is situated within a dense, singular-layered beech (Fagus *sylvatica* L.) forest, in Pian del Cansiglio (46° 3' 19'' N 12° 22' 51'' E, 1100 m a.s.l.) located on the Eastern Alps, Italy, about 50 km north-east of the Po valley. The soil is calcareous, nutrient-rich, and relatively moist Haplic Luvisol (WRB). The climate is oceanic, with a mean annual temperature of 7.2 °C and a mean annual precipitation of 1767 mm. The forest stand has a tree density of 168 trees per hectare, with the trees aged between 130-140 years (Teglia et al 2022). The average annual atmospheric N deposition rates measure 17.7 kg ha-1 y-1 in open-field conditions and 12.6 kg N ha-1 y-1 via throughfall Cecchini et al (2021).

3.3.2. Experimental design

Four N addition treatments were established in 2015: control (N0, receiving only ambient deposition), soil additions of 30 (N30) and 60 kg N ha^{-1} yr^{-1} (N60), and canopy addition of 30 kg N ha^{-1} yr^{-1} (N30A). The experiment followed a completely randomized design, including three replicate plots for each treatment. These plots, measuring 30 x 30 m, are positioned adjacently. To prevent any lateral contamination effects, intensive sampling and plant measurements were conducted within the central area (15 x 15 m) of each plot.

The N30 and N30A treatments of 30 kg N ha^{-1} y^{-1} were considered for mimicking the effects of a realistic increase in N deposition, which was slightly above the ambient deposition in the area, which was 20 kg N ha^{-1} y^{-1} when the experiment started. Moreover, the dose of 60 kg N ha^{-1} y^{-1}

aimed to highlight the forest sensitivity to N additions, intending to drive the ecosystem toward a state of N saturation.

N was applied using an ammonium nitrate (NH_4NO_3) solution and N isotopic composition of the fertilizer is -0,833 ‰. For direct-to-soil treatments (N30 and N60), a backpack sprayer was utilized to add the solution (35.1 g L^{-1}). In the case of above-canopy fertilization (N30A), the solution (4.5 g L^{-1}) was dispersed above the tree canopy using a circular sprinkler (r=21 m) installed on the central tree within the plots. N addition started in May 2015, and since then, treatments have been performed three times per year during the growing season.

3.3.3. Sampling collection

The sampling campaign, conducted in July 2018, occurred four years after the treatments started. Fresh, sun-exposed leaves were sampled from three distinct trees within each plot (n=36; see Teglia et al 2022 for details). Leaves were washed, oven-dried at 60 °C until constant weight and ground into powder. Within the intensive area of each plot, two trees were considered to collect fine roots, soil, and differentiate organic layers (partially undecomposed litter – OF, and decomposed litter – OH), by following this protocol: at 1 m distance from the stem base of each tree, three equidistant points were identified for sampling. Organic layers (OF and OH, 1 cm depth) and soil cores (5 cm diameter and 10 cm depth, excluding the organic layers) were collected and pooled per each sampled tree (n=24). Roots were collected from the same points around the trunk base and pooled together, creating a comprehensive sample representative of the tree's fine root system (n= 24). Finally, in autumn, after trees shed their leaves, beech fruits were collected from the 5 litter collectors per plot, and pooled together (one sample per each plot, n=12).

3.3.4. Sample preparation and chemical analyses

In the laboratory, litter layers (OH and OF) and a soil subsample, sieved with a 2 mm mesh sieve, were oven-dried at 60 °C until constant weight and ground, while the remaining soil subsamples were kept at -20 °C for genetic analysis. Fine roots (<2 mm in diameter) were detached from coarse roots, cleaned with sterile distilled water over a 100 µm mesh sieve, and then stored at -20 °C until analysis. Fine roots were placed in Petri dishes containing distilled water to distinguish ECM root tips (ERT) from ECM-free fine roots. Under a stereomicroscope (Carl Zeiss 475003 – 9902), meticulous removal of residual soil and organic particles was conducted using fine-tips forceps. Dead and dry root fragments and tips were discerned from living ones and were excluded from further processing. The ERT, and the ECM-free root tips were counted, allowing the calculation of the ERT percentage (fraction of ERT to total viable root tips) for each sample. Approximately 62 %

were found to be colonized and there were no significant differences across N treatments. Following this, the fine roots were sectioned into pieces using a sterile blade, while all ERT were carefully separated just above the fungal mantle to produce ERT samples (for method details see Scartazza et al 2023). Both ERT and fine roots samples were dried at 60 °C until reaching a constant weight and ultimately ground into powder. Fruit capsules were discarded and only seeds were dried at 60 °C until constant weight, weighted and ground to powder.

The total N concentration and the stable N isotopic composition ($\delta^{15}N$) were analyzed using an elemental CHN analyzer coupled with an isotope-ratio mass spectrometer (CF-IRMS, Delta Plus Thermo Fisher, Bremen, Germany). Samples, weighted in tin cups with varying amounts (approximately 1.5 mg for soil and fine roots, between 0.5-0.7 mg for the remaining matrices). Isotopic values are expressed in the delta (δ) notation as a relative deviation from the international standard (Fry 2006) according to the equation:

$$\delta^{15}N(‰) = \left(\frac{R_{sample}}{R_{standard}} - 1\right) x\ 1000 \qquad \text{Eq. 1}$$

Where R_{sample} and $R_{standard}$ are the ^{15}N and ^{14}N isotope ratios of the sample and the international standard (i.e., the atmospheric N_2: 0.0036782), respectively.

3.3.5. Quantification of functional genes related to soil N processes

Microbial DNA was extracted from 0.25 g of soil using the DNeasy PowerSoil Kit (Qiagen Inc., USA) according to manufacturer's protocol. Following extraction, DNA concentration was quantified using the Qubit® 3.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) double-stranded DNA (dsDNA) high-sensitivity (HS) assay. Quantitative PCR (qPCR, real time PCR) method was used to quantify the genes involved in nitrogen fixation (nifH), nitrification (ammonia-oxidizing archaeal, amoA AOA, ammonia-oxidizing bacterial, amoA AOB and nitrite-oxidazing bacterial, nxrB), denitrification (Nitrobacter nxrB, nirK, nirS, nos Z, qnorB and nirK fungi). Sequence primers, aneling temperature, bp of each target gene were showed in Table S1. See supplementary materials for details on the methodology applied.

The abundance of genes was expressed with logarithm 10 of the number of copies per gram (g) of soil, normalized both for microbial DNA extracted and soil dry weight.

3.3.6. Data analysis

The evaluation of $\delta^{15}N$ differences ($\Delta\delta^{15}N$, where the Δ indicate the offset) between close compartments within the forest was conducted using the simplified N cycle scheme in **Figure**. These offsets were computed to assess the influence of N addition on N uptake and translocation processes. Positive values denoting an enrichment in ^{15}N from the preceding to the subsequent compartment, while negative values indicated ^{15}N depletion. The calculations were performed using the following equations:

$$\Delta\delta^{15}N_{(soil-E\ )} = |\delta^{15}N_{soil}| - |\delta^{15}N_{ERT}| \quad \text{Eq. 2}$$

$$\Delta\delta^{15}N_{(LitterOH-ER\ )} = |\delta^{15}N_{LitterOH}| - |\delta^{15}N_{ERT}| \quad \text{Eq. 3}$$

$$\Delta\delta^{15}N_{(ERT-Fine\ roots)} = |\delta^{15}N_{ERT}| - |\delta^{15}N_{Fine\ roots}| \quad \text{Eq. 4}$$

$$\Delta\delta^{15}N_{(Fine\ roots-Leave\ )} = |\delta^{15}N_{Fine\ roots}| - |\delta^{15}N_{Leaves}| \quad \text{Eq. 5}$$

$$\Delta\delta^{15}N_{(Leaves-LitterOF)} = |\delta^{15}N_{Leaves}| - |\delta^{15}N_{LitterOF}| \quad \text{Eq. 6}$$

$$\Delta\delta^{15}N_{(LitterOF-LitterOH)} = |\delta^{15}N_{LitterOF}| - |\delta^{15}N_{LitterOH}| \quad \text{Eq. 7}$$

$$\Delta\delta^{15}N_{(LitterOH-Soil)} = |\delta^{15}N_{LitterOH}| - |\delta^{15}N_{soil}| \quad \text{Eq. 8}$$

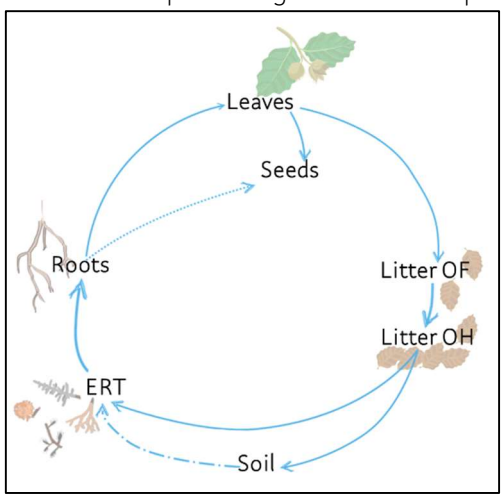

Figure 1: Simplified N cycle scheme which includes the forest ecosystem components measured: soil, ERT, fine roots, leaves, seeds, litter OF, and litter OH.

The seeds compartment was excluded from offset calculation due to the absence of replicated data per tree. Additionally, the enrichment factor (EF = $\delta^{15}N_{leaves}$ - $\delta^{15}N_{soil}$), often used as an indicator of ecosystem N status (Emmett et al 1998; Amundson et al 2003), was calculated.

All data were averaged within the treatment, considering individual trees as replication units, except for seeds data, where the plot served as the replication unit. Dixon's test was utilized to detect outliers, which were then excluded from the analyses if identified; moreover, Bartlett's test was employed to assess the homogeneity of variance before the analysis. One-way analysis of variance (ANOVA) was employed to detect differences in mean N concentration (%), $\delta^{15}N$, $\Delta\delta^{15}N$, and functional genes among treatments (N0, N30A, N30, N60) within individual forest compartment. A posteriori separation of the means was performed by the Student Newman-Keuls test (SNK).

Structural Equation Modeling (SEM) was employed to investigate the effect of N fertilization on roots N uptake. SEM are probabilistic models that unite multiple predictors and response variables in a single complex causal network to test different scenarios of hypothesized causal relationships (Bollen, 1989; Lefcheck, 2016; Shipley, 2016). The characteristic of SEM analysis makes them more suitable for describing a system and predicting causal relationships when compared to conventional univariate analyses (Grace, 2008). The SEM model was constructed based on

established relationship from previous studies in the literature concerning N pathways from soil to roots. The model was performed separately for each treatment (N0, N30A, N30, N60) to obtain regression coefficients related to the N addition regime. The level of significance (p-value) was set to ≤ 0.05.

All statistical analyses were conducted using R open-source software (R Core Team, 2017) and the following specific packages: lavaan (Yves 2012), agricolae (de Mendiburu and Yassen, 2020), Outliers (Komsta 2011), easyanova (Arnhold 2013), ggplot2 (Wickham, 2016), Tidyverse (Wickham et al, 2019).

3.4. Results

3.4.1. Relationships between N concentration and $\delta^{15}N$ across forest samples

A significant positive correlation between N and $\delta^{15}N$ was observed across all studied organic compartment (Adj R^2: 0.353, p<0.001, **Figure**, panel h), except for fine roots (p: 0.054; **Figure**, panel c), while N and $\delta^{15}N$ in soil were negatively related (Adj R2: 0.80, p< 0.001, **Figure**, panel i). Within each forest compartment, the strongest relationship was observed in ERT (Adj R^2: 0.60, p< 0.001; **Figure**, panel b), whereas inorganic soil exhibited the strongest negative relationship (Adj R^2: 0.42, p< 0.001; **Figure**, panel g). Notably, there were no changes in the relationships between N and $\delta^{15}N$ across the various compartments due to N treatments.

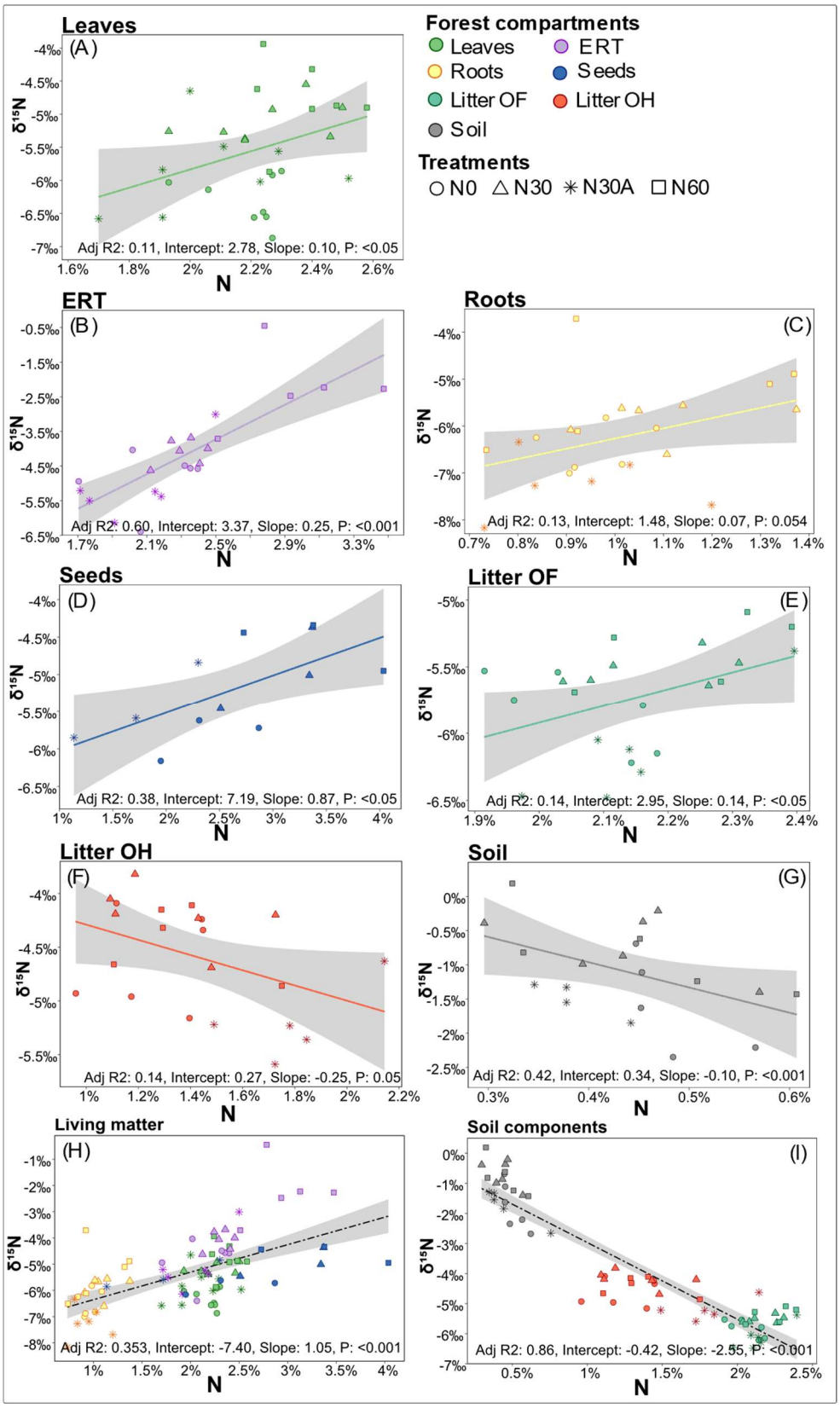

Figure 2: Linear relationships between N isotope composition ($\delta^{15}N$) and N concentration across different forest compartments: leaves (a), ERT (b), fine roots (c), seeds (d), litter OF (e), litter OH (f), soil (g), all living matter together (h), and soil components (i). Each point represents a single value referred to the tree units for all the compartments, except for seeds where the sample units were the plots. Different shapes and colors indicate the treatment applied and the compartments, respectively, as reported in the legend. The correlation coefficient (Adj R2) and significance level (p) are reported.

3.4.2. Changes in N concentration and $\delta^{15}N$ in relation to the forest compartment and treatment

Four years after the onset of fertilization, we did not detect any significant difference in N concentration between the control and all N fertilized plots in the case of leaves, fine roots, litter OF, and soil (**Figure**, panel a). Notably, N30 and N60 treatments had a positive effect on N concentration in seeds (+29.4% for N30, and +42.3% for N60, p: 0.025, **Figure** panel a), and in ERT, where an increase of +38.4 % was found only in the case of the higher dose (N60, $p<0.001$, **Figure** panel a). Conversely, the N30A fertilization affected N concentration only in the case of the litter OH, which increased by 45.9 % compared to the control (p: 0.002, **Figure** panel a).

The direct-to-soil fertilization treatments substantially affected N isotopic composition across the investigated compartments. Remarkably, plots treated with the N60 dose showed significant enrichment in ^{15}N in all analyzed forest compartments, with the only exception of the litter OH (see **Figure**, panel b). The substantial increase in $\delta^{15}N$ was particularly evident in ERT, leaves, and seeds, revealing increments of 53.9 %, 24.2 %, and 21.5 %, respectively, compared to the control plots. Similar results were also observed in N30 plots, although differences from N0 were statistically significant only for leaves (+18.6 %), ERT (+15.3 %), and soil (+60.3 %). Conversely, the effect of N30A treatment on $\delta^{15}N$ was less pronounced (**Figure**, panel b). Similarly to what was observed in the case of N30 and N60 treatments, the above-ground forest components (i.e., leaves and seeds) were more enriched in $\delta^{15}N$ compared to the control, though the difference was not significant. In the case of below-ground compartments (i.e., litter layers, ERT, and roots), samples were generally more depleted in ^{15}N (i.e., more negative $\delta^{15}N$ values) in N30A plots than control plots, except for soil $\delta^{15}N$, where the mean values were similar across treatments. However, a statistically significant difference between N30A and N0 was observed only in the case of fine roots (-12 %, **Figure** panel b).

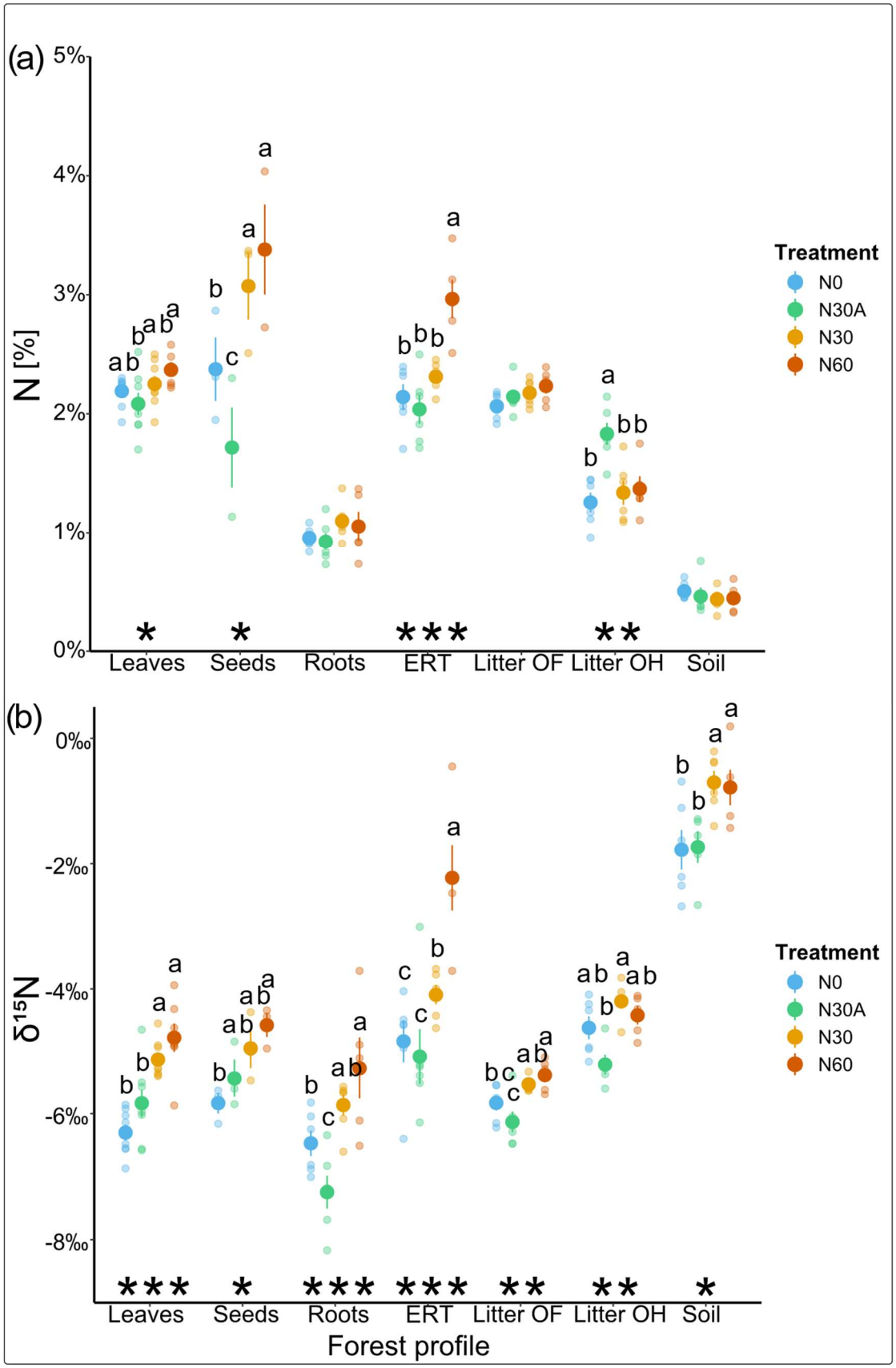

Figure 3: Mean values (and standard errors) of N concentration (%) (panel a) and isotopic composition ($\delta^{15}N$ ‰) (panel b) across different forest compartments for each of the four treatments (indicated with different colors). Different letters indicate significant differences (* p<0.05, ** p<0.01, *** p<0.001) in the mean values among different treatments and within each compartment as a result of the ANOVA and the SNK test.

3.4.3. Soil N cycling-related functional genes

The fertilization treatments showed no significant effect on N cycling-related functional genes (NFGs) in the soil (**Figure**), except for nosZ ($p<0.05$), which, however, did not show a clear treatment effect. Except for qnorB, all the bacterial denitrifiers (nirK: 9.20±0.2, nirS: 7.33±0.2, and nosZ: 7.67±0.2 - log_{10} copies/g) were more abundant than fungal denitrifiers (nirK: 6.77±0.3 log_{10} copies/g, $p<0.001$; **Figure**). Particularly, the abundance of bacterial nirK genes was the highest among all the investigated soil functional genes.

Regarding nitrification, we observed that archaeal nitrifiers (amoA AOA: 7.68±0.1 log_{10} copies/g) were significantly higher than bacterial nitrifiers (amoA AOB: 6.88 ±0.1 log_{10} copies/g). Moreover, bacterial nitrifiers involved in the nitrite to nitrate step of nitrification were significantly higher than bacterial involved in the first step of nitrification (amoA AOB). Finally, functional genes related to N_2 fixation (nifH) were the least abundant (6.65±0.3 log_{10} copies/g) in the soil (Figure 4).

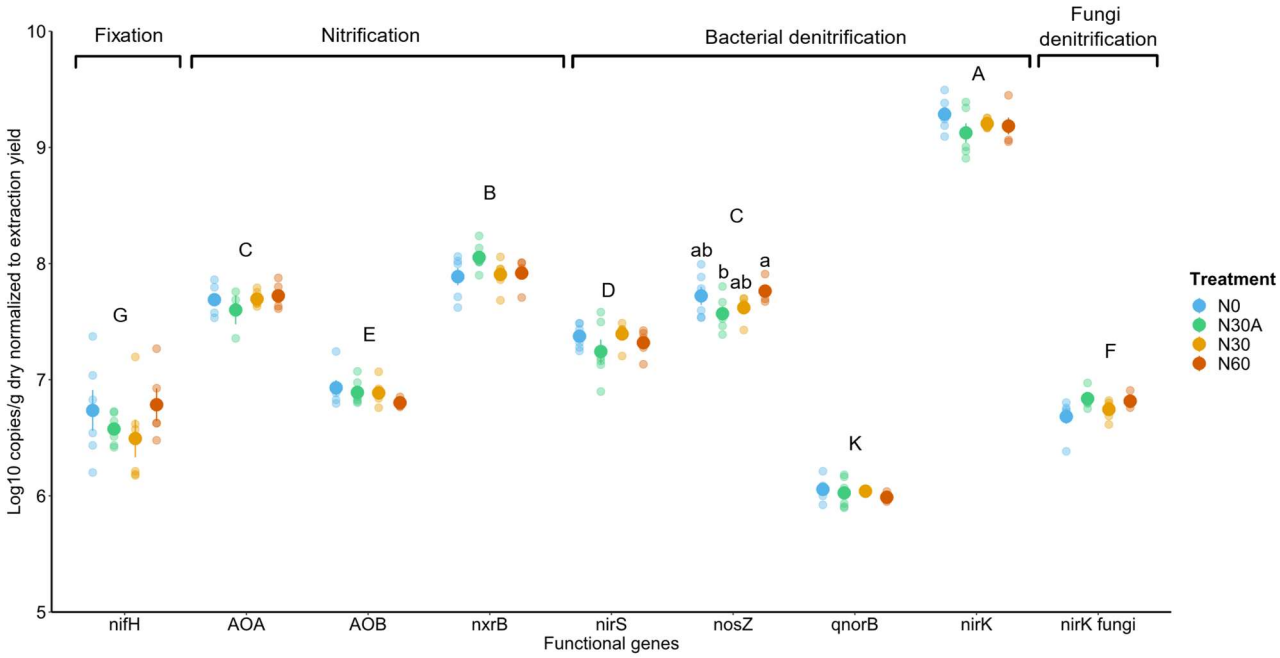

Figure 4: Abundance of N cycling-related functional genes in soil: nifH (nitrogenase gene), AOA and AOB (archaeal and bacterial amoA ammonia monooxygenase genes), nxrB (nitrite oxidoreductase genes), nirS and nirK (nitrite reductase genes), nosZ (nitrous oxide reductase genes), qnorB (nitric oxide reductase), and nirK fungi (fungal nitrite reductase genes). Colors designate Colors designate distinct treatmens, detailed in the legend. Capital letters indicate statistical differences across soil functional genes, while lowercase letters denote differences within treatments for each functional gene (SNK test).

3.4.4. Enrichment factor and offset in $\delta^{15}N$ between compartments

The data in **Table 1** includes the Enrichment factor (EF) and offsets between compartments ($\Delta\delta^{15}N$). The EF exhibited a ranged between -5.45 and -3.18 ‰. Although the treated plots generally

displayed higher EF values than the N0 plots, this increase was not statistically significant. Nevertheless, the N fertilization significantly altered the relative ^{15}N enrichment from one compartment to the next along the key path of N cycle (Figure 1). In comparison to soil and litter OH, ERT tissues exhibited a greater enrichment in ^{15}N in the N60 treated plots. In the control plots, $\Delta\delta^{15}N_{(LitterOH-ERT)}$ values were nearly zero (-0.22 ± 0.3 ‰), indicating a relatively consistent $\delta^{15}N$ between the two compartments. Conversely, in the N60 plots, the $\Delta\delta^{15}N_{(LitterOH-ERT)}$ was 2.19 ± 0.5 ‰, indicating that ERT tissues were more enriched in 15N than the organic substrate. A contrasting pattern emerged in the pathway from ERT to fine root tissues, where the depletion in ^{15}N from ERT to the fine roots compartment was notably higher in N60 plots (-3.04 ± 0.2 ‰) compared to N0 plots (-1.64 ± 0. 2 ‰). While a noticeable ^{15}N enrichment from fresh leaves to partially decomposed litter (OF) was evident in N0 plots (0.48 ± 0.2 ‰), the $\Delta\delta^{15}N_{(Leaves-LitterOF)}$ in treated plots (N30A, N30, and N60) significantly differed from the control ($p<0.001$). Indeed, negative $\Delta\delta^{15}N$ values were observed in fertilized plots, meaning a consistent depletion in ^{15}N in litter OF compared to fresh leaves (-0.37 ± 0.2 ‰, -0.46 ± 0.1‰, and -0.71 ± 0.2 ‰ for N30A, N30, and N60, respectively). Additionally, only the N30A treatment significantly affected the $\Delta\delta^{15}N_{(Fine\ roots-Leaves)}$ values (1.48 ± 0.2 ‰, p: 0.008), which denoted a greater ^{15}N enrichment in leaves from fine roots compared to the N30, N60, and N0 plots.

Table 1: Enrichment Factor (EF) and offset ($\Delta\delta^{15}N$) among subsequent forest compartments. Each value represents the mean ± standard error (n=6). Significant differences ($p<0.05$) are highlighted in bold. Letters indicate differences within the rows (SNK test).

	N fertilization treatments				
	N0	N30A	N30	N60	p-values
Enrichment Factor (‰)					
EF	-4.52 ± 0.3	-3.87 ± 0.3	-4.36 ± 0.3	-3.81 ± 0.4	ns
$\Delta\delta^{15}N$ (‰)					
Soil - ERT	-3.06 ± 0.4 b	-3.32 ± 0.6 b	-3.39 ± 0.3 b	-1.44± 0.7 ab	**0.048**
LitterOH - ERT	-0.22 ± 0.3 b	-0.29 ± 0.3 b	0.10 ± 0.2 b	2.19 ± 0.5 a	**<0.001**
ERT - Fine roots	-1.64 ± 0.2 a	-2.17 ± 0.4 a	-1.77 ± 0.2 a	-3.04 ± 0.2 b	**0.017**
Fine roots - Leaves	0.17 ± 0.2 b	1.48 ± 0.2 a	0.80 ± 0.1 b	0.37 ± 0.6 b	**0.008**
Leaves - Litter OF	0.48 ± 0.2 a	-0.37 ± 0.2 b	-0.46 ± 0.1 b	-0.71 ± 0.2 b	**<0.001**
LitterOF - LitterOH	1.21 ± 0.2	0.86 ± 0.2	1.33 ± 0.1	0.95 ± 0.2	ns
LitterOH-Soil	2.84 ± 0.2	3.49 ± 0.2	3.50 ± 0.2	3.54 ± 0.2	ns

3.4.5. SEM analysis

The structural model employed in the SEM analysis is depicted in **Figure**, and results are presented in **Table 2**. The analysis revealed a significant and direct association between $\delta^{15}N$ in ERT and $\delta^{15}N$ in fine roots, consistently significant across the treatment groups ($p<0.05$, $p<0.01$, $p<0.05$, and $p<0.001$ in N0, N30A, N30, and N60, respectively). The regression coefficients varied from 0.45 in N30A, to 0.74 in N60 plots. In N0 plots, soil $\delta^{15}N$ exhibited a direct correlation with the litter OH layer (coeff: 0.83, $p <0.001$). However, this relationship was not significant in any of the treated plots. Within the N30A fertilization treatment, soil $\delta^{15}N$ influenced both the ERT (coeff: 0.36, $p<0.001$) and fine roots $\delta^{15}N$ (coeff: 0.88, $p<0.001$), while $\delta^{15}N$ of litter OH showed a negative correlation with $\delta^{15}N$ of ERT (coeff: -1.1, $p<0.001$). Conversely, N0 and both N30 and N60 fertilized plots denoted a direct relationship between litter OH and ERT $\delta^{15}N$ values, though it was significant only in N60 plots (coeff: 0.64, p:0.034).

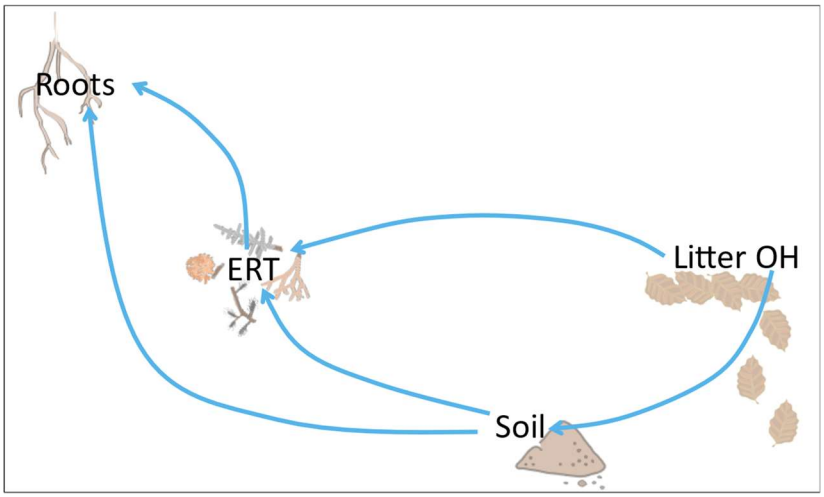

Figure 5: SEM model structural scheme, delineating the N pathways from soil to roots.

Table 2: Standardized regression coefficients (Coeff) and p-values (p) for relationships among $\delta^{15}N$ values measured in the belowground forest compartments. Data were elaborated through a SEM analysis, following the N pathways delineated in **Figure**. Data reported referred to each fertilization treatment (N0, N30A, N30, and N60). Significant regression coefficients ($p<0.05$) are highlighted in bold.

	$\delta^{15}N$ Soil ~ $\delta^{15}N$ Fine roots		*$\delta^{15}N$ ERT ~ $\delta^{15}N$ Fine roots*		*$\delta^{15}N$ Lett OH ~ $\delta^{15}N$ ERT*		*$\delta^{15}N$ Soil ~ $\delta^{15}N$ ERT*		*$\delta^{15}N$ Litter OH ~ $\delta^{15}N$ Soil*	
	Coeff	*p*	*Coeff*	*p*	*Coeff*	*p*	*Coeff*	*p*	*Coeff*	*p*
N0	-0,13	0.664	**0,73**	**0.013**	0,45	0.513	-0,12	0.855	**0,83**	**<0.001**
N30A	**0,88**	**<0.001**	**0,45**	**0.006**	**-1,1**	**<0.001**	**0,36**	**<0.001**	0,46	0.243
N30	0,56	0.077	**0,66**	**0.038**	0,19	0.613	-0,38	0.313	0,04	0.919
N60	**-0,40**	**0.001**	**0,74**	**<0.001**	**0,64**	**0.034**	-0,52	0.088	0,18	0.680

3.5. Discussion

3.5.1. Does N additions (regardless of the approach) increase N availability?

Contrary to expectations, four years of fertilization treatments—whether above-canopy or direct-to-soil—did not substantially change the N concentration among the majority of the compartments analysed. Indeed, was not detected a significant increase in N concentration in leaves, fine roots, litter, or soil. This aligns with findings from Da Ross et al (2023), who similarly reported no differences in N content across these ecosystem compartments following the addition of 20 kg N $ha^{-1}y^{-1}$, applied both above and below the canopy in a temperate deciduous forest. Consistent with these results, Zhang et al (2015) also found no observable differences in leaf nitrogen content after the addition of 25 or 50 kg N $ha^{-1}y^{-1}$, both above and below the canopy in their manipulative study in a subtropical deciduous forest.

We expected to observe a more rapid response of N concentration in the tree compartments – such as leaves, seeds, and fine roots - with the N30A treatment compared to the N0 and N30 treatments. The basis for this expectation lies in the hypothesis that the N uptake mechanism has the potential to meet up to 30% of the annual growth requirement in specific trees (Friedland et al) and is likely to be as important as root uptake, especially in N limited sites (Ferraretto et al 2022). Additionally, studies on nursery stock and young forest trees, including works by Eilers et al. (1992) and Hättenschiwiler and Körner (1998), have demonstrated increased above-ground biomass in response to canopy N additions. Indeed, we observed a higher foliar biomass production in the N30A plots, suggesting that this increase may contribute to the dilution of the canopy N budget, potentially obscuring any discernible difference in foliar N concentration (Ravaioli et al 2022). Another hypothesis proposed by Dail et al. (2009) suggests that the greater amount of N retained by canopies may not necessarily result from direct plant uptake and immediate utilization of the additional nitrogen. Instead, their observations indicate that, in the short term, most of the retained N resides on plant surfaces—such as twigs, branches, and stems—potentially remaining unavailable for plant uptake for several years (Dail et al 2009).

A contradictory trend emerged in the response of the seeds N concentration. While both doses of the direct-to-soil N fertilization (N30 and N60) led to a significant increase in seeds N concentration, the N30A treatment exhibited a significant decrease in seed N concentration compared to the control plots. In the N30 and N60 treatments, an increase in fruit production was also observed (data not shown). This, coupled with the higher N concentration found in seeds compared to leaves, might suggest that fruit production is a highly nutrient-demanding process

that could take advantage of the additional N supply, at the tree level (Han et al 2017). Several studies (Hoch and Keel 2006; Miyaki et al 2007; Muller-Haubold et al 2015) pointed out that beech trees preferentially allocate N to fruit production during the masting years. However, we acknowledge that our results rely on a limited number of replicates (three per treatment) for seed samples. Nevertheless, they provide indications of the effects of nitrogen deposition on the dynamics of nutrient allocation to seed production that would certainly need to be further explored at this experimental site.

The belowground N concentration compartments exhibited limited impact from all fertilization treatments. While we expected a general increase in N concentration in all the belowground compartments, we did not anticipate that this would be detected only for the ERT compartment, under the higher dose of soil fertilization treatment (N60). The absence of substantial variations in N concentration within both tree and soil compartments could be partially attributed to the initially high N availability at the forest site. This observation aligns with findings from leaf physiological and nutritional traits in the same manipulative experiment, as reported by Teglia et al (2023). Their results indicate that the forest stand is characterized by a eutrophic condition, where N is not a growth-limiting factor but rather a potential stress factor. Despite the absence of direct measurements and quantification of N losses from the ecosystem in the present study, it is plausible to consider that if the additional N exceeds the forest ecosystem demand, the excess N could be susceptible to loss through mechanisms such as leaching or volatilization.

3.5.2. $\delta^{15}N$ as a better indicator than N concentration of changes in N dynamics induced by above-canopy and soil fertilization

While the absence of substantial changes in N concentrations introduces uncertainty regarding potential alterations in ecosystem N cycling, the clear response of $\delta^{15}N$ to fertilization undeniably highlights the significant impact of additional inputs of inorganic N on the N cycle within the ecosystem.

Under increasing levels of N availability and N deposition, plant tissues are expected to show higher $\delta^{15}N$ values (Templer et al 2007; Craine et al 2015), while soil might present a decreasing trend in $\delta^{15}N$ (Craine et al 2015). Notably, irrespective of the treatment applied, $\delta^{15}N$ values displayed a positive correlation with nitrogen concentrations in plant compartments and decreased in the soil with an increase in N concentration. This observation confirmed the usefulness of $\delta^{15}N$ as a valuable indicator for depicting changes in N dynamics within an ecosystem.

Isotopic signature in a given tree compartment is affected by the soil $\delta^{15}N$ and isotope fractionations during N uptake and metabolism within plants (Högberg 1997; Craine et al 2015). If those processes have a similar influence – no matter the fertilization approach, we should observe the same N dynamics in above-canopy vs direct-to-soil fertilizations. Yet, this was not the case in our study. Both N30 and N60 treatments resulted in an elevated N concentration and more positive $\delta^{15}N$ values in the ERT and foliar tree compartments as well as in the soil compared to the control. Conversely, in the N30A plots, neither N concentration nor $\delta^{15}N$ values in ERT and soil differed significantly from the N0 plots.

The higher N concentration in the ERT for the N30 and N60 treatments suggests a potential role of ectomycorrhizal fungi in the immobilization of N derived from direct-to-soil fertilization. Moreover, the lack of a concurrent increase in N concentration in fine root and foliar tissues might indicate that the excess N immobilized in the ERT compartment may not be proportionally transferred to the host plants. Nevertheless, the mechanism by which ectomycorrhizal fungi balance N availability between transferring N to the host plant and meeting their own N demand remains unclear. Nasholm et al (2013) hypothesized that the N addition to the soil should override the N demand of mycorrhizal fungi, reducing the proportion of N they immobilize and thereby increasing the supply of N to the tree canopy. However, the N concentration and isotopic composition do not appear to fully support this hypothesis. This suggests that fertilization may have altered the dependence of trees on mycorrhizal association. While we cannot exclude the possibility of changes in the composition of ectomycorrhizal fungi species related to N uptake, which can only be assessed using molecular analyses, SEM analysis results demonstrated a high correlation between $\delta^{15}N$ in ERT and $\delta^{15}N$ in roots across all treatments. This confirms the essential role of ectomycorrhizal association for beech soil N-uptake, regardless of the soil N availability condition. The N30A treatment showed low values of $\delta^{15}N$ in the below-ground organic compartments, with statistically significant differences observed only in fine roots compared to the N0. Notably, the ^{15}N depletion observed in the decomposed litter (OF layer) in comparison to the N30 treatment, coincided with a higher N concentration, distinguishing significantly both from N0 and directly soil fertilized plots. Interestingly, the elevated N concentration in litter OH did not originate from N enrichment in partially decomposed litter (OF), where no discernible differences were observed among all treatments. These findings suggest that the N30A fertilization may affect litter quality (e.g., increase in lignin to cellulose ratio) with a likely inhibitory effect on litter decomposition (Morrison et al 2018). Indeed, the negative effect of added N on decomposition rates has been extensively documented in the literature (Knorr et al 2005, Janssen and Luyssaert

2009), particularly in high-lignin litter types, which is generally the case of mature beech forest (Sariyildiz and Anderson 2003; Trap et al 2013), attributed to a reduction in microbial extracellular enzyme activity, that may respond adversely to N addition (Magill and Aber 1998, Sinsabaugh et al 2002; Knorr et al 2005; Morrison et al 2018). Conversely, the ^{15}N depletion in fine roots may suggest the extensive enzymatic capabilities of ectomycorrhizal fungi, enabling host plants to access N from fresh, litter-derived sources that are less enriched (Hobbie et al 2008). Alternatively, it has been proposed that ectomycorrhizal fungi sequester sufficiently ^{15}N-enriched N and transfer to their host plants N depleted in ^{15}N (Oimette et al 2013; Hogberg et al 1997), which is then reflected in more negative $\delta^{15}N$ values for trees with ECM associations than for non-mycorrhizal trees (Craine et al 2009). In both scenarios, the depletion of ^{15}N in fine root tissues in N30A was explained by a strong dependency of plants on ectomycorrhizal fungi activity for N uptake.

3.5.3. Does N fertilization change the abundance of microbes involved in soil N processes?

While direct measurements of nitrification and denitrification were not carried out at the site, we employed molecular analyses to quantify functional genes directly involved in these processes. This indirect approach allows to assess whether various fertilization approaches resulted in differential soil N losses.

Analysis of the NFGs data revealed that the abundance of microbes associated to key N processes, i.e., N fixation, nitrification, and denitrification, remained largely unaffected by the various N treatments applied. Despite this, the abundances of NFGs mirrored those typically found in nitrogen-rich forest soils characterized by low soil C/N ratios (averaging 12.12 in our study site – data not shown), predominantly hosting ectomycorrhizal-associated tree species and exhibiting an accelerated N cycle (Tang et al 2018, Shi et al 2023). This characterization was corroborated by contrasting our observed NFG abundances with those documented in prior studies on forests ecosystems housing deciduous ectomycorrhizal-associated tree species, revealing that the majority of our measured NFG abundances were one to two orders of magnitude higher than those reported in the literature (Shi et al 2023, Ernfors et al 2011; Singavarapu et al 2023). The relatively low abundance of N-fixing bacteria NFG might indicate that the soil microbes community invested less energy in N-fixing processes. Previous studies in the literature reported the preference of N-fixing bacteria for soil with high C/N ratios, and their activity might diminish under elevated N atmospheric deposition (Levy-booth et al 2014; Wallestien et al, 2006; Hallin et al, 2009).

Conversely, NFGs associated with N-loss processes (e.g. nirK, nxrb, AOA) were relatively abundant (regardless of the treatment), entailing a high potential for denitrification and nitrification rates in the soil. Within the NFGs involved in denitrification processes, the nirK bacterial nitrite reductase gene emerged as the predominant, exhibiting abundances two orders of magnitude greater than those of the nirK fungi, highlighting the limited contribution of the fungal community to the potential denitrification rates. Concerning nitrification, our results showed the dominance of the archaeal amoA gene (AOA) in ammonia oxidation, the first and rate-limiting step of autotrophic nitrification (Frijilink et al 1992). Both AOA and AOB had relatively high abundances, denoting a high potential nitrification rate at the site, with AOA showing a comparatively higher abundance than AOB. The observed relative abundances of AOA and AOB in our experiment closely mirror those documented by Zhang et al (2015) in a N manipulative experiment conducted in a subtropical deciduous forest including both above and below-canopy N additions with similar doses considered at our site (25 and 50 kg N $ha^{-1}y^{-1}$). The authors reported a significant reduction in AOA and AOB abundances, particularly with higher application doses, when employing the above-canopy fertilization approach. In contrast, the below-canopy treatment only reduced AOA abundances with the higher dose. However, our results do not seem to corroborate this observed reduction in nitrifier abundances, at least not four years after the experiment started.

Overall, the NFGs data suggested a relatively low potential for N fixation, but high potential rates for denitrification and nitrification. These findings hint that although fertilization treatments did not alter the soil microbial activity, the microbial N demand might already be met, prompting a reduction in N storage processes. This could potentially lead to increased N losses from the forest ecosystem, such as gaseous N emissions and nitrate leaching (Niu et al 2006; Levy-booth et al 2014).

3.5.4. Do tree N pathways and N-uptake strategies change across the fertilization treatments?

The enrichment factor (EF) is often used as an integrated measure of N cycling processes in the soil (Emmett et al, 1998; Amundson et al 2003). In the present study, the inability of EF to detect any differences among the fertilization treatments applied confirmed that the simple comparison of $\delta^{15}N$ between plant foliage and soil N cannot always provide clear evidence of possible alteration in N plant-soil dynamics (Handley and Scrimgeour 1997; Evans 2001; Robinson 2001). Indeed, plant $\delta^{15}N$ is the result of multiple factors, such as the sources of N (i.e. soil, precipitation, N_2

fixation, N canopy uptake), the forms of soil N used, the influences of mycorrhizal symbioses, fractionations during and after N uptake by plants and plant phenology (Hogberg et al 1997). For instance, in the N60 plots, we observed a significant enrichment in ^{15}N-N ERT compared to the ^{15}N-N in litter OH, which was not obvious in the other treatments. Furthermore, the results of the SEM analysis highlighted varying relationships between ^{15}N-N in litter OH and ^{15}N-N ERT in ERT across the different treatments. Interestingly, the relation between these two compartments was significant only for the N30A and the N60 treatments, albeit with different directions (positive in the case of N60, negative in the case of N30A). These results suggest that direct-to-soil and above-canopy treatment may differently affect the plant N-uptake strategy, potentially altering the primary soil N source (e.g. organic or inorganic N), depending on the soil availability of inorganic forms of N. When moving to the next pathway (ERT to fine root) more negative values were observed in N60 treatment, which is in line with what we would expect. Indeed, ectomycorrhizal fungi transfer preferably ^{15}N-depleted N to trees (Amundson et al, 2003; Hobbie & Ouimette, 2009), especially in high N availability conditions.

Leaves were consistently enriched in ^{15}N-N compared to fine root tissues, with the N30A plots exhibiting a significantly higher enrichment compared to the N0 and both direct-to-soil treatments. Evans at al (1996) reported that this enrichment pattern is common in plants utilizing NO_3^- as their N source (such as beech trees; Templer and Dawson 2004; Leberecht et al 2016). This is attributed to the NO_3^- reduction processes occurring during plant assimilation and translocation. However, the distinct disparity in the ^{15}N-N enrichment in leaves observed in the N30A treatment may be attributed to an increased foliar N uptake, facilitated by the above-canopy fertilization method. Furthermore, looking at the $\Delta\delta^{15}$N between fresh leaves and litter OF, appeared evident that both above canopy and direct-to-soil treatments determined a change in the N resorption mechanism before the leaf shading. Indeed, while N litter OF appeared enriched in ^{15}N compared to the fresh leaves in control plots, the opposite pattern was observed in all the fertilized plots, regardless of the methodology of application.

3.6. Conclusions

In conclusion, our study highlights the complexity inherent in utilizing natural N isotope abundances to elucidate changes in N dynamics within forest ecosystems. These dynamics result from intricate interactions among soil, microbes, plants, and environmental factors such as temperature and precipitation. Although the use of natural ^{15}N abundances posed challenges in clearly assessing and quantifying forest ecosystem N retention vs. losses under different

fertilization approaches, natural $\delta^{15}N$ emerged as a more reliable and sensitive indicator of forest N cycling compared to N concentration. Alterations in $\delta^{15}N$ provided valuable insights into nitrogen ecosystem dynamics, emphasizing the varying impacts of different fertilization approaches on forest nitrogen cycling. Despite the site was already N enriched, as evidenced by previous studies (Teglia et al, 2022) and NFGs analysis, we did not observe clear signals of N saturation, contrasting with observations from previous soil fertilization experiments in natural ecosystems (Magill et al 2000; Ochoa-Hueso et al 2013; Bergkvist and Folkeson 1991). The higher dose of soil N fertilization revealed an incipient alteration of the N cycle, yet N concentrations were minimally affected by the treatment. After four years of simulating realistic increases in N deposition on an already N-rich site, our research suggests, at least in the short term, the absence of strong signals of N saturation, but potential development of stress response in plant (Teglia et al 2022), without any significant positive impact on C uptake (Ravaioli et al 2022). Therefore, extending the study to assess long-term responses to N additions would be valuable to detect any development in the ecosystem N saturation condition.

3.7. Acknowledgments

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3.8. Supplementary materials

3.8.1. Quantitative PCR protocol

Single PCR reactions were prepared in a total volume of 10 µl containing the following: 5 µl of KAPA SYBR® FAST qPCR Master Mix (Roche), 0.25 µl of forward and reverse primer (10 µM) (Metabion); 0.25 µl of dimetyl sulfoxide, DMSO, (Sigma); 1,5 µl H2O, and 2.5 µl template DNA (4 ng μl^{-1}). qPCR was performed by using LightCycler 480 (Roche). At the end of each run, melting curve analysis of the PCR products was conducted to confirm that the fluorescence signal came from specific PCR products and not from primer-dimers or other artifacts. Additionally, an agarose gel (2%) was run to check the correct size of amplicons.

The qPCR standards were obtained from the following sources: soil gDNA (bacterial *amoA*, *qnorB* and *nirk*Fungi); environmental clone N3-16 [AB62272] (archeal *amoA*); *Acidithiobacillus ferrooxidans* (*nifH*); *Nitrospira* sp. (*nxrB*); *Sinorhizobium melioti 1021* (*nirK* and *nosZ*) and *Ralstonia eutropha H16* (*nirS*) The PCR amplified DNA from the soil samples and the cultured microorganisms were purified using mi-Gel Extraction Kit (Metabion, Germany) and then standard curves were generated based on quantified PCR products with a series of 1:10 dilutions ($R^2 \geq 0.99$ for each gene). All the samples and standards were analyzed in triplicate and several negative controls were included. Amplification efficiencies were calculated as E = [10(−1/slope) -1]*100, with the following results: *nifH* 93% ; bacterial *amoA* 95%; archeal *amoA* 93%; *nxrB* 96% ;*nirK* 98%; *nosZ* 92%; *nirS* 89% ; *qnorB* 92% and *nirK*Fungi 94%.

Table S1: Sequence primers, aneling temperature, bp of each target gene

Target gene	Primers	Sequence	Tº Annealig	Gene bp	Reference
nifH	nifHF	AAAGGYGGWATCGGYAARTCCACCAC	55	458	Rosh et al 2002
	nifHR	TTGTTSGCSGCRTACATSGCCATCAT			
Archeal amoA	amo19F	ATGGTCTGGCTWAGACG	55	624	Leininger et al 2006
	amo643R	TCCCACTTWGACCARGCGGCCATCCA			Treusch et al 2005
Bacterial amoA	amoA1F	GGGGTTTCTACTGGTGGT	60	500	Rotthauwe et al 1997
	amoA1R	CCCCTCKGSAAAGCCTTCTTC			
Nitrobacter nxrB	nxrB1F	ACGTGGAGACCAAGCCGGG	58.5	411	Vanparys et al 2007
	nxrB1R	CCGTGCTGTTGAYCTCGTTGA			
nirK	nirK876C	ATYGGCGGVCAYGGCGA	58.5	164	Harter et al 2014
	nirK1040	GCCTCGATCAGRTTRTGGTT			
nirS	nirScd3aF	AAC GYS AAG GAR ACS GG	57	413	Michotey et al (2000)
	nirSR3cd	GAS TTC GGR TGS GTC TTS AYG AA			Throback et al (2004
nosZ	nosZ2F	CGC RAC GGC AAS AAG GTS MSS GT	65-60 (TouchDown)	257	Henry et al 2006
	nosZ2R	CAK RTG CAK SGC RTG GCA GAA			
qnorB	qnorB2f	GGN CAY CAR GGN TAY GA	55	263	Kim 2020
	qnorB5r	ACC CAN AGR TGN ACN ACC CAC CA			
Fungal nirk	nirKfF	TACGGGCTCATGTAYGTNSARCC	54	480	Wei et al 2015
	nirKfR	AGGAATCCCACASCNCCYTTNTC			

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4. Application of multiple stable isotope approach to study water and nutrients relations in the tropical mountain cloud forest ecosystems

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4.1. Summary

Tropical Montane Cloud Forests (TMCFs) are unique ecosystems, situated in narrow altitudinal bands, act as "water towers" and are particularly vulnerable to global change drivers. Factors such as increased temperatures, altered water regimes, and atmospheric pollution pose threats to TMCFs integrity. In this context, a recently established research project collaboration, between the University of Bologna and the University of Costarica, focuses on the Monteverde TMCF in Costa Rica, aiming to assess water and nutrient dynamics across the atmosphere-plant-soil continuum comprehensively. This article presents preliminary results on carbon (C) and nitrogen (N) concentrations and isotopic compositions ($\delta^{13}C$ and $\delta^{15}N$) in leaves and soil. The objective was to test water-use efficiency (WUE) and N balance in four tree species, each exposed to two distinct light and humidity conditions within the canopy. We found that foliar N concentration variability was mainly attributed to tree species, underscoring the species-specific nature of nutrient dynamics. Canopy exposure and tree species insufficiently explain foliar $\delta^{15}N$ variability, but they play pivotal roles in influencing foliar $\delta^{13}C$ values and thus water use efficiency (WUE). The remarkably low foliar $\delta^{13}C$ values underscored the strong dependence of this forest ecosystem on water availability, confirming the potential impact of alterations in precipitation frequency and in cloud/mist formation dynamics. Future research will integrate repeated monthly sampling campaigns to comprehensively assess water and nutrient dynamics, incorporating meteorological data and measurements of fog/rainfall/tree water isotopes and air $\delta^{13}C$. This holistic approach will allow us to deepen our understanding of TMCFs and their responses to changing environmental conditions, contributing valuable insights for their conservation.

4.2. Introduction

Tropical forests play a pivotal role in providing a diverse array of ecosystem services, including climate and water regulation (Borma et al., 2022). Approximately 55% of the forest's carbon (C) is estimated to be stored in tropical ecosystems, making them crucial natural solutions for mitigating climate change (Pan et al., 2011; Le Quéré et al., 2018). Among these ecosystems, Tropical Montane Cloud Forests (TMCFs) stand out for their essential role in water regulation, acting as "water towers" for lowland areas (Bubb et al., 2004; Viviroli et al., 2007).

Situated in narrow altitudinal bands between 800 and 3500 meters on mountains in Africa, America, Asia, and equatorial Oceania, TMCFs are unique ecosystems characterized by persistent cloud cover and mist at the vegetation or ground level (Bubb et al., 2004; Kappelle 2004; Bruijnzeel et al., 2005). This atmospheric condition ensures regular contact between tree canopies and cloud water (Ray et al., 2013). The development and structure of TMCFs are strongly influenced by trade winds, with 'leeward cloud forests' exhibiting well-developed characteristics and taller trees compared to their windward counterparts at the same elevation (Bruijnzeel, 2005). The distinctive climate conditions of TMCFs lead to abundant moss and epiphyte (e.g. orchids, ferns, bromeliads) cover on branches and stems, with regular cycles of cloud formation playing a crucial role in shaping and sustaining these ecosystems (Bruijnzeel, 2005; Whitmore, 1998). For these reasons, TMCFs might be particularly vulnerable to global change drivers, including land use transformation, increased temperatures, and altered water regimes, which have the potential to pose substantial threats to the integrity and resilience of TMCF ecosystems (Dietterich et al., 2022). Still et al (1999) simulated environmental conditions for such forests at doubled CO_2 doubling concentration and found that a combination of reduced cloud contact and increased evapo-transpiration could threaten the persistence of these ecosystems. Moreover, recent reports indicate an elevation in cloud base height, potentially reducing cloud and fog occurrence in the lower altitude levels of TMCFs (Karmalkar et al, 2013; Foster 2001; Williams et al, 2015). Given the vital role of cloud and fog in influencing evapotranspiration rates and hydrological balances, such changes could have profound consequences leading to an increase in tree mortality, changes in species distribution, and stream dynamics, thus compromizing the ability of TMCFs to provide essential hydrological services (Bittencourt et al., 2019; Bittencourt, 2014; Martin and Bellingham, 2016).

Another factor threatening TMCFs is atmospheric pollution, with particular reference to anthropogenic activities like agricultural practices and fossil-fuel combustion, which may impact nutrient availability in TMCFs by releasing increasing amounts of reactive nitrogen (N) compounds into the atmosphere. These compounds, transported by fog, could deposit onto natural

ecosystems, potentially altering the nutrient dynamics in TMCFs. While TMCFs are considered more N-limited compared to lowland tropical forests, the rise in N availability is expected to significantly affect tree and associated microbes and ephypites functionality, potentially altering the C to N balance in these ecosystems (Wood et al., 2019; Melillo et al., 2002).

Thus, understanding key ecophysiological and ecohydrological processes underpinning C, water, and nutrient cycles in TMCFs is essential to better predict TMCFs functioning under global change as well as put in place policy action to preserve their important ecosystem services.

The application of stable carbon and oxygen isotopes in plant tissues can provide important information on tree water-use efficiency (Ehleringer et al. 1993; Fanquhar et al. 1989, Guerrieri et al. 2019) and changes in the water sources for trees and their relative contribution at the intra-seasonal scale (e.g. Dawson et al. 2002 and reference therein), respectively. Moreover, nitrogen isotope composition can offer the opportunity to link ecophysiological information derived from $\delta^{13}C$ with changes in nitrogen availability (Craine et al. 2009). The isotope approach has been extensively used in temperate and boreal forests, while its application is limited in highly complex and diverse tropical ecosystems (see global analyses by Diefendorf et al. 2011; Basu et al. 2021). For instance, studies conducted in the Amazon forest in Brazil (Ometto et al. 2006) and tropical dry forest in Costa Rica (Leffler and Enquist 2002) showed high variability among species and between dry and wet season for foliar $\delta^{13}C$ values, which was attributed to the complex forest structure and associated changes in light conditions and contribution of recycled CO_2 coming from soil respiration. Moreover they suggest a high functional diversity in relation to environmental conditions, with particular reference to changes in water availability between dry and wet season (Leffler and Enquist 2002).

Here we present preliminary results from a study conducted in the Monteverde TMCF (Costa Rica, Figure 1), which was part of a recently established collaboration between the Meteorology and atmospheric Physics research group of the University of Costa Rica and the Forest ecology research group of the University of Bologna. The overarching goal of the collaboration was to comprehensively assess water and nutrients dynamics across the atmosphere-plant-soil continuum within the Monteverde TMCF. Specific goals addressed in this study were: 1) Elucidate the role of fogs and rains as plant water source and their role in affecting plant water-use efficiency by integrating measurements of fog/rainfall $\delta^{18}O$ and air $\delta^{13}C$ with $\delta^{18}O$ in tree water and $\delta^{13}C$ plant tissues;

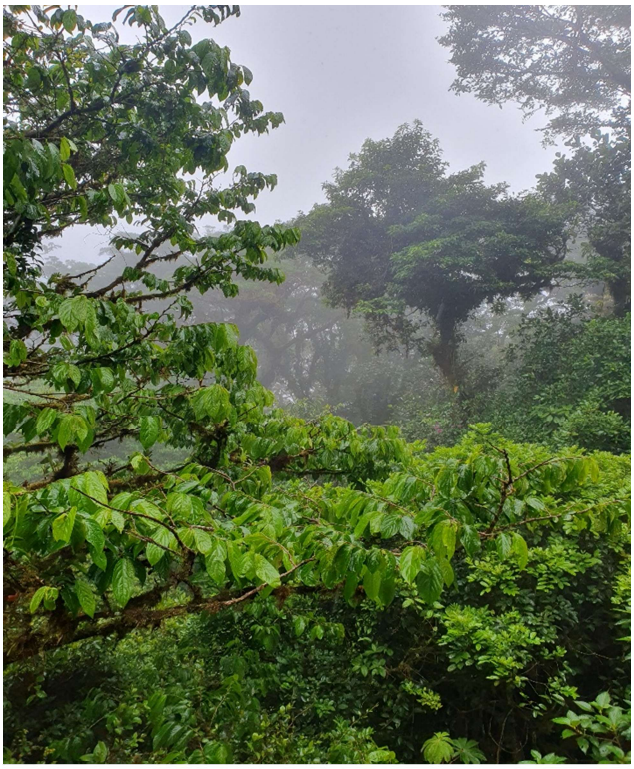

Figure 1: Photo taken at the site by Teglia A., capturing the rich biodiversity of the forest ecosystem in the Monteverde Cloud Forest.

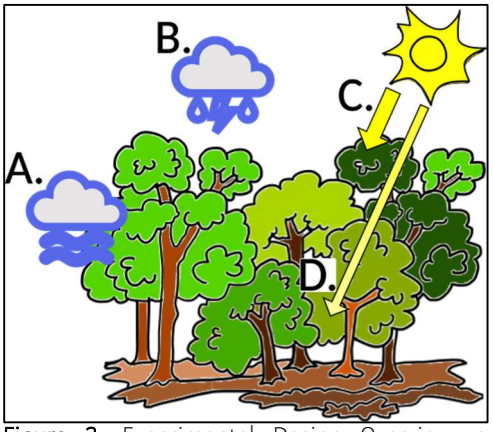

Figure 2: Experimental Design Overview: a graphical representation illustrating the experimental conditions within the forest ecosystem. Two distinct tree conditions are depicted: sun-exposed trees, receiving full sunlight and water primarily from precipitation (C and B); and canopy-shaded trees, situated beneath the forest canopy and receiving water not only from precipitation but also from mist (A and D).

2) Assessing the role of fog as a potential N input to plants through the foliar nutrient uptake, by using N concentration and N isotopic compositions in soil and foliar samples. To achieve these goals, a comprehensive monthly field sampling campaign has been planned, started in May 2023 and concluding in May 2024 to undertake intensive collection of air, fog and precipitation water samples, as well as soil and plant tissues. In this dissertation, we show preliminary results on C and N concentrations and isotopic compositions in leaves and soil as obtained on samples collected during the first sampling campaign (May 2023) I participated to. The aim is to test WUE and N balance in four different tree species, each exposed to two distinct light and humidity conditions within the canopy, following the conceptual scheme presented in figure 2.

4.3. Material and Methods

4.3.1. Site description

The research was carried out within the primary Monteverde Cloud Forest Reserve (10°18' N, 84°48' W, 1500 m asl), situated on the Cordillera de Tilaràn in northern Costa Rica. The forest thrives on persistently moist soils derived from volcanic rhyolites, categorized as Typic Dystrandept (Vance and Nadkarni, 1990). The forest's structural composition varies with wind exposure, featuring taller trees (25-30 m) on leeward slopes and smaller trees on windward slopes (Bruijnzeel, 2005). Additionally, towards the exposed crests of the windward slopes, the vegetation height diminishes further to 3-10 m across an altitudinal gradient of only 30-50 m (Lawton and Dryer, 1980). The forest comprises a well-developed subcanopy layer (Nadkarni et al., 2004), especially in the leewars slopes. The forest tree density, calculated by measuring trees with a diameter above 10 cm at breast height, is 555 trees/ha (Nadkarni et al., 2004). The elevated humidity levels and shaded understories have fostered the proliferation of diverse and abundant epiphytes (Ingram and Nadkarni, 1993; Nadkarni et al., 2000). Three distinct seasons are delineated primarily by the seasonal migration of the intertropical convergence zone: 1) a dry season (mid-January to April), marked by moderate northeasterly tradewinds and wind-driven clouds and mist; 2) a rainy season (May to November), characterized by convective precipitation during the afternoons and evenings, with a high frequency of extreme weather events, particularly hurricanes, in September, October, and November; and 3) a transition season (late November to mid-January), distinguished by strong tradewinds and wind-driven precipitation and mist (Clark et al., 2000). The mean annual precipitation (over 2018-2022 time period) measured at the Monteverde Cloud Forest Reserve meteorological station, located near the Ventana panoramic viewpoint, was 2739 mm. Mean monthly minimum and maximum temperatures (for the same time period) fluctuated between 10 and 14.8 °C and 17.6 and 26.8 °C, respectively. The diurnal temperature range averaged 4.4 °C. Air relative humidity consistently exceeded 80%, maintaining an annual average of 97.3%. Detailed data on precipitation, average temperature, and relative humidity are depicted in Figure 3, presented on both a monthly and daily basis.

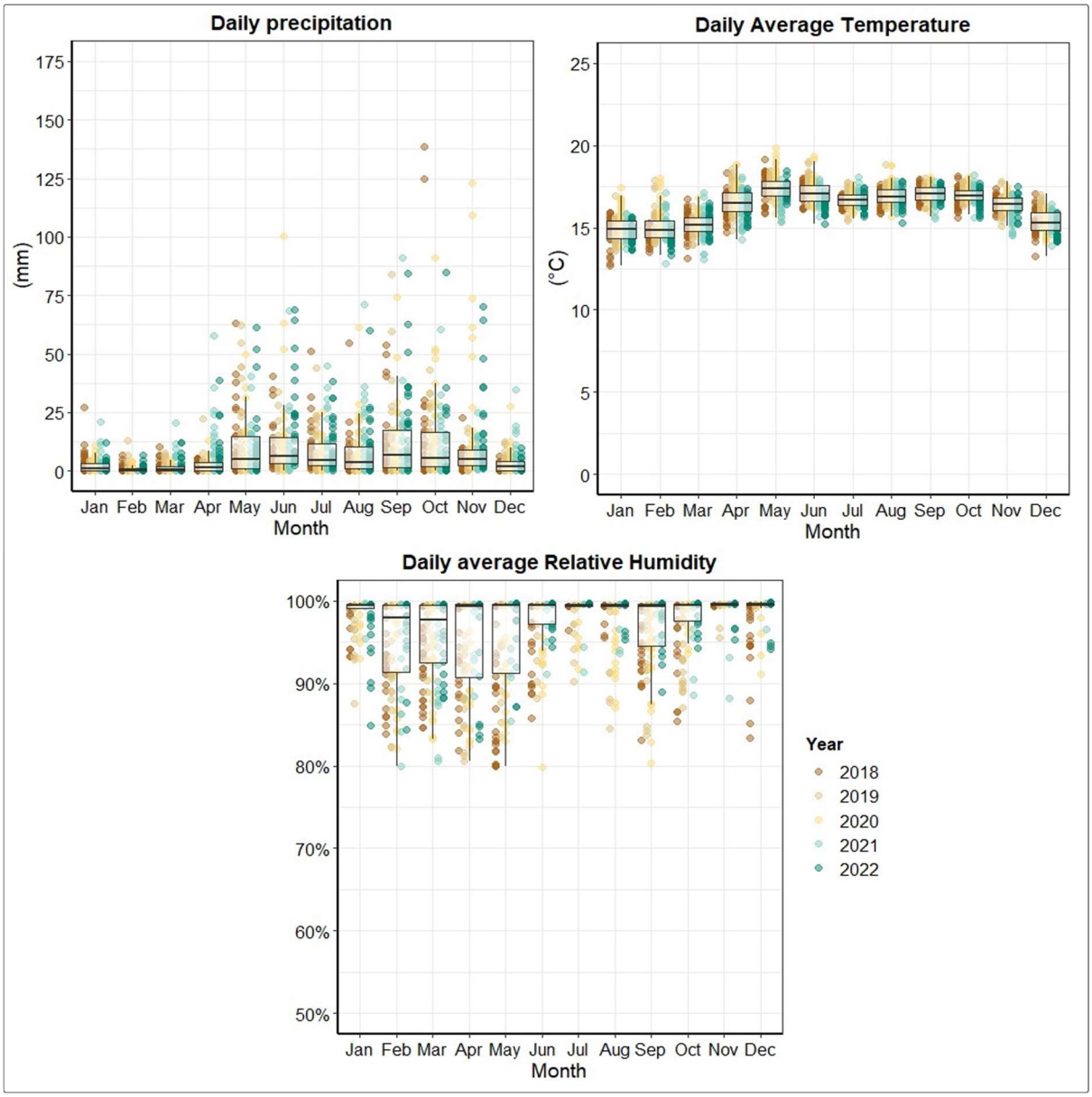

Figure 3: Temporal variability in meteorological parameters at Monteverde Cloud Forest Biological Reserve: Daily precipitation (top left), daily mean temperature (top right), and daily average relative humidity (bottom). Each point is the daily average value of a given parameter recorded over 2018-2022 time period. Box plots represents the distribution and central tendencies of each parameter for every month over the specified time period.

4.3.2. Experimental design

We chose four distinct tree species for our study: Ocotea *insularis*, Guatteria *oliviformis*, Hampea *appendiculata*, and Conostegia *oerstediana* (foliar morphology illustrated in Figure 4). The selection of these species was based primarily on their distribution within the forest and secondarily on the availability of prior literature reporting their physiological characteristics or C isotopic composition in tree tissues.

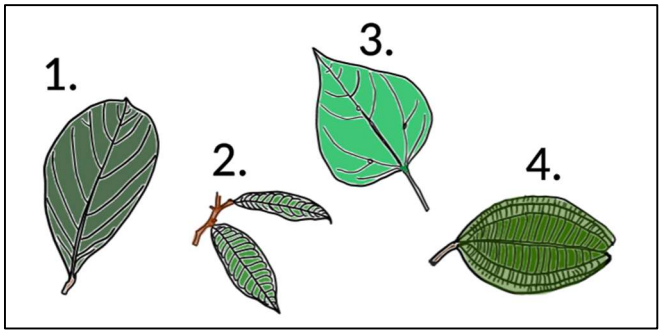

Figure 4: Foliar morphology of the four tree species selected. 1. *Ocotea insularis*, 2. *Guatteria oliviformis,* 3. *Hampea appendiculata*, 4: *Conostegia oerstediana*. These images are for illustrative purpose only, and the figures are not presented with a precise scale.

The individual trees were identified and targeted walking through the "Sendero Nuboso (A)", "Sendero Camino", and "Sendero Ventana" paths, starting from the Reserve's information point and culminating at the "Ventana" panoramic viewpoint (refer to the Reserve's map in Figure 5). For each selected species, four individuals were selected - two exposed to direct sunlight, and other two in shaded position within the canopy. This way was possible to account for different light conditions experienced by the selected trees.

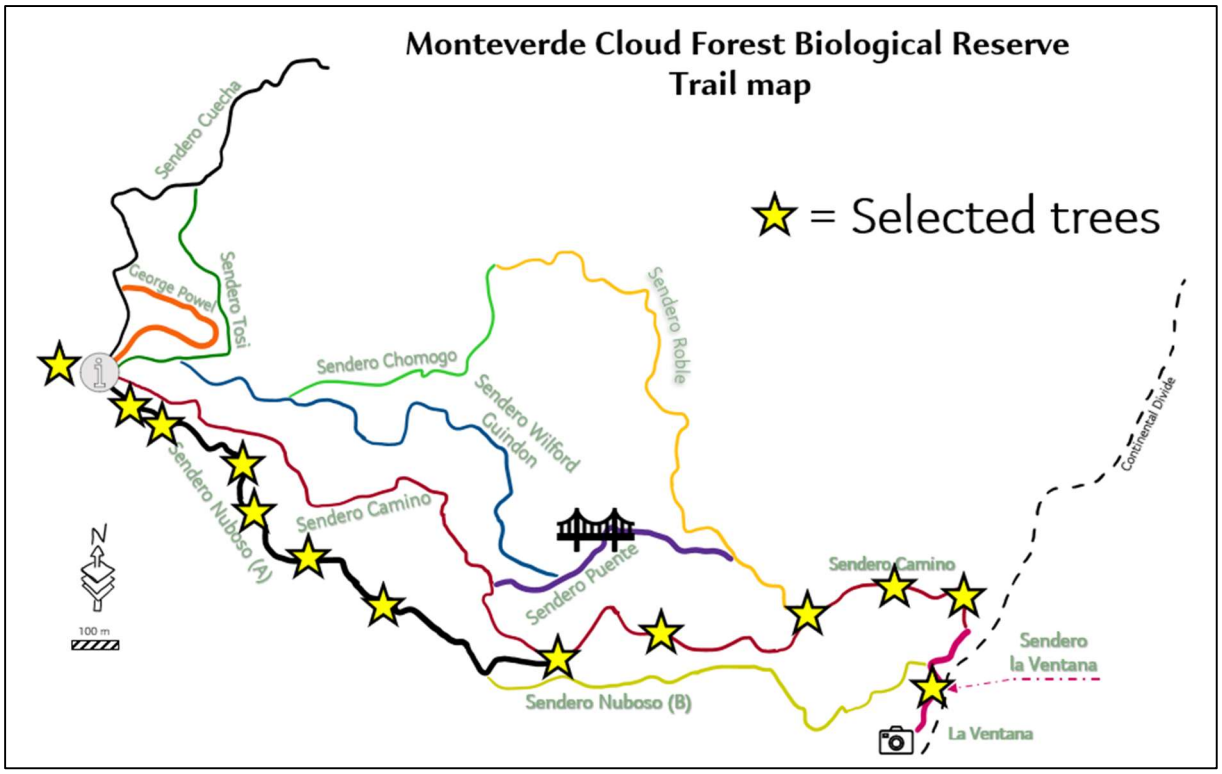

Figure 5: Monteverde Cloud Forest Biological Reserve Trail map. Yellow stars indicate the location of the selected trees for the sampling.

4.3.3. Tree and soil sampling

The sampling campaign, conducted on May 11, 2018, coincided with an unexpectedly dry period at the site, attributed to a delay in the onset of the regular rainy season, which typically starts on the first week of May.

From each individual tree (n=16), one branch with 5-10 leaves was sampled using telescopic tree pruner, enabling access to leaves up to 5 m in height. Subsequently, a subsample of two leaves was carefully stored in glass-threaded vials with caps to preserve their water content. These vials were maintained in a cooler bag during fieldwork and later stored at 4 °C in the laboratory until sample preparation and chemical analysis. The remaining leaves were stored in paper bags, and once back in the laboratory, they were oven-dried at 60 °C until achieving a constant weight, and used for subsequent calculations of leaf area and leaf mass per area.

At a distance of 1 m from the base of each tree, three equidistant points were identified for soil core extraction, utilizing cores with a diameter of 5 cm and a depth of 15 cm. Soil cores surrounding the same tree were pooled and an aliquote of c.a. 3 g was stored in glass vials with caps, kept in a cooler bag during fieldwork, and subsequently preserved at 4 °C in the laboratory until sample preparation for chemical analysis.

4.3.1. Chemical analyses

Foliar and soil samples underwent a cryogenic water extraction methodology, serving the dual purpose of obtaining the dry matter (for chemical analyses) and enabling the collection of the water contained in the samples (for oxygen isotope analyses). The dried foliar and soil samples were ground into a powder, and approximately 1.5 mg of soil and 1.0 mg of leaves were weighed into tin capsules. The total C and N concentration and the relative stable isotope composition ($\delta^{13}C$ and $\delta^{15}N$) were analyzed using and elemental CHN analyzer coupled with and isotope-ratio mass spectrometer (CF-IRMS, Delta Plus Thermo Fisher, Bremen, Germany).

4.3.2. Statistical analyses

Data were analyzed in a complete randomized factorial design with two factors: species (4 levels: *Ocotea insularis*, *Guatteria oliviformis*, *Hampea appendiculata*, and *Conostegia oerstediana*) and canopy position (2 levels: sunlight exposed and shade leaves). When the two-way analysis of variance (2-way-ANOVA) showed a significant interaction between factors ($p \leq 0.05$), *a posteriori* separation of means was performed by the Student Newman-Keuls test (SNK). All statistical

analysis were carried out with the R open-source software (R Core Team 2019) using the *easyanova* (Arnhold 2013), *agricolae* (de Mendiburu and Yassen, 2020), and *ggplot2* (Wickham, 2016) packages.

4.4. Results and Discussion

4.4.1. Soil C and N concentration and isotopic composition

The soil C percentage exhibited a wide range between 10.98% and 31.23% across all forest sampling points. The elevated C percentage can be attributed to the thickness of the organic soil horizon at the site, with an average depth of 15 cm observed at all sampling points. These findings align with the soil data reported by Watanable et al. (2023) in the organic horizon of tropical forests. However, a substantial variability in soil C concentration was evident among sampling points, with a notably higher average concentration under sunlight-exposed canopy tree conditions compared to shaded ones (24.2% vs. 18.7%, p: 0.0314). In contrast, the variability in soil $\delta^{13}C$ values, ranging from -31.1‰ to -28.1‰, was less pronounced and did not show significant variation under different light exposure or tree species. These low $\delta^{13}C$ values are consistent with the C isotopic composition of the top forest floor in tropical forests, as documented in previous research in Costa Rican tropical rainforest soils (Powers and Schlesinger, 2002). Typically, $\delta^{13}C$ values are relatively more negative in soil organic horizons and progressively increase with soil depth (Powers and Schlesinger, 2002). More negative $\delta^{13}C$ in the top soil could be explained by the isotopic signature of the source the organic carbon (shed leaves), which in turns reflect tree physiological responses to environmental factors but also the contribution of more depleted atmospheric CO_2 derived from the fossil fue combustion (the so-called Suess effect, Francey et al. 1999). A significant negative relationship between soil C concentration and $\delta^{13}C$ values (Adj R^2: 0.46, p: 0.001, Figure 6.A) was observed, which reflects changes in soil C dynamics (Ma et al. 2012; Garten et al, 2000). Indeed, lower %C could be the result of higher decomposition, during which lighter ^{12}C is preferred over the heavier isotope, thus leading the remaining soil organic matter more enriched in the ^{13}C, and hence with more positive $\delta^{13}C$ values (Elheringer et al. 2000).

The C:N ratio in the forest topsoil averaged 13.83 ± 2.2, with N concentration ranging between 0.7% and 1.9%. The observed C:N ratios and N concentrations did not suggest N limitation for the site, as values above 20 are indicative of potential N limitation (Springob and Kirchmann, 2003).

A positive correlation between soil $\delta^{13}C$ and $\delta^{15}N$ (Adj R^2: 0.77, $P < 0.001$; Figure 6.B) was observed, indicating that higher natural abundance of the ^{13}C isotope in soil C was associated with increased

enrichment of the ^{15}N isotope in soil N. This result indicates a coordination between C and N isotope fractionations during decomposition and mineralization (Nell et al. 2018). This correlation aligns with findings by Peri et al. (2011) in Patagonia's native forests, who suggested that the strong C and N cycling relation in forest ecosystems might result from efficient cycling of N through soil organic matter, especially in soil moisture conditions, associated with relatively little or neglectable N losses from the ecosystem.

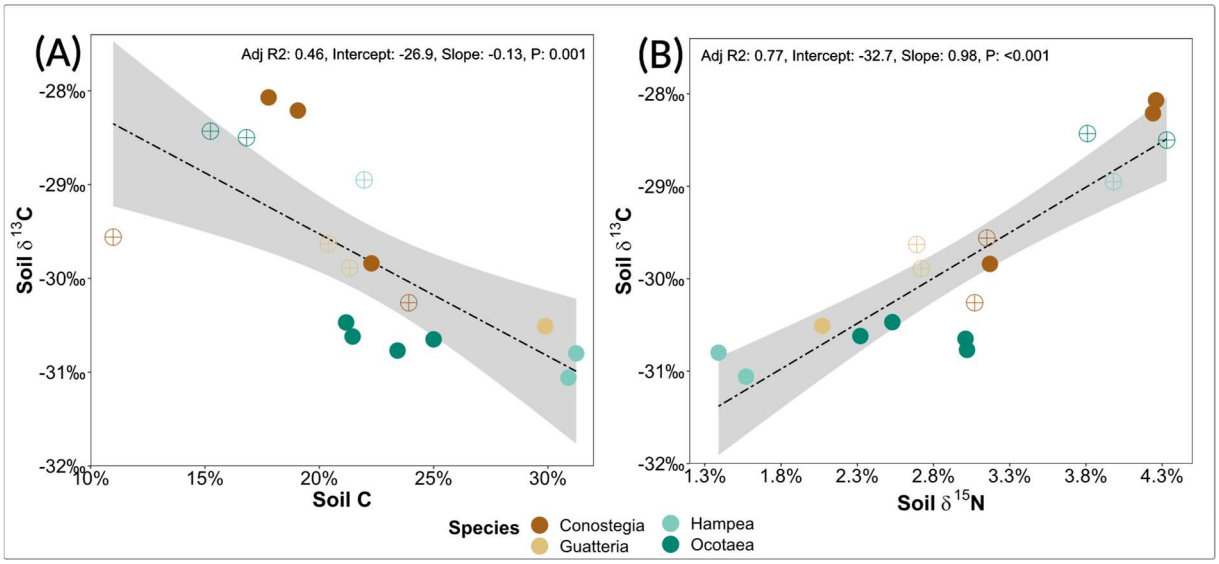

Figure 6: Relationships between soil C isotope composition ($\delta^{13}C$) and soil total C concentration (panel A), and soil N isotopic composition ($\delta^{15}N$), panel B. Each point represents a single value referred to the tree units. Different colors indicate the tree species, as reported in the legend. Crossed circles referred to full sunlight trees, while filled circle referred to canopy-shaded trees. Statistics from the linear regression analyses are reported, i.e., the regression coefficient (Adj R^2) and significance level (p).

4.4.2. The influence of tree canopy exposure and species on water use efficiency

The influence of tree canopy exposure and species on WUE was investigated to understand how these factors contribute to the water-use strategies of trees in the studied ecosystem. WUE is a crucial parameter that reflects the efficiency by which plants use water to perform photosynthesis and produce biomass. Leaf $\delta^{13}C$ can be used to assess changes in intrinsic WUE (iWUE), that is the ration between photosynthesis and stomatal conductance (Elheringer et al. 1993, Farquhar et al. 1989). Indeed, a plant showing a high $\delta^{13}C$ (less negative) values, indicates lower discrimination against the heavier ^{13}C isotopes during diffusion of CO_2 through stomata and photosynthesis, related to either a reduction of stomatal conductance under no changes in photosynthesis or an increase in photosynthesis under small changes in stomatal conductance (Scheidegger et al. 2000). In both cases, the result is that plant shows a high WUE. Under moist conditions (like the case of TCMFs), we can assume no stomatal limitation, thus differences among species in $\delta^{13}C$ (and hence

iWUE) mostly reflect differences in photosynthesis due to light conditions (within a species) and/or physiological strategies (across species).

Global analyses showed that highest values of foliar carbon isotope discrimination (and then $\delta^{13}C$ lowest) were generally measured in tropical rain forests compared to temperate ecosystems (Diefendorf et al. 2010). However, it should mentioned that in this analysis there was no data for TCMFs and in general for tropical ecosystems in central and southern America, pointing to an important data gap that will need to be filled. The $\delta^{13}C$ values observed in leaves from both light exposed and shaded trees, ranging between -38.2 and -30.1‰, align closely with the foliar $\delta^{13}C$ values reported for trees in the understory canopy layer of a tropical rainforest in Panama by Graham et al. (2014). Graham et al. (2014) noted this range of foliar $\delta^{13}C$ values for canopy trees receiving from 0 to 40% of the available light. We should acknoweledge that the trees sampled in this study had a maximum height of 5 m, and it is likely that both the sunlight-exposed and shaded trees were influenced to some extent by the shading effects of dominant trees in the upper canopy layer. The low $\delta^{13}C$ values observed in both light exposure conditions may indicate slower photosynthetic rates associated with shade conditions (Santiago and Wright, 2007). Additionally, the increased stomatal conductance related to high humidity, particularly prevalent in the understory and mid-canopy layers at the site, could contribute to these lower values (Ometto et al 2006). Moreover, the "canopy effect" could also contribute to explaining negative $\delta^{13}C$ values. This effect involves the fixation of CO_2 more depleted in ^{13}C in the understory layer, attributed to microbial respiration activity in the soil (Medina and Michin, 1980; van der Merwe and Medina 1989). The presence of this canopy effect could be more important in in tropical ecosystems, with high complexity forest structure compared to temperate and boreal forests, and thus should be considered in the interpretation of isotopic data in tropical forest ecosystems.

Despite the dominant canopy layer might have partially shaded the light-exposed trees, observed foliar $\delta^{13}C$ data differed significantly between the two tested canopy position, showing higher foliar $\delta^{13}C$ in sunlight-exposed trees compared to the shaded ones (-34.24 vs -35.60 ‰, p: 0.001). This result confirmed that the percentage of light received differed between trees in different canopy positions.

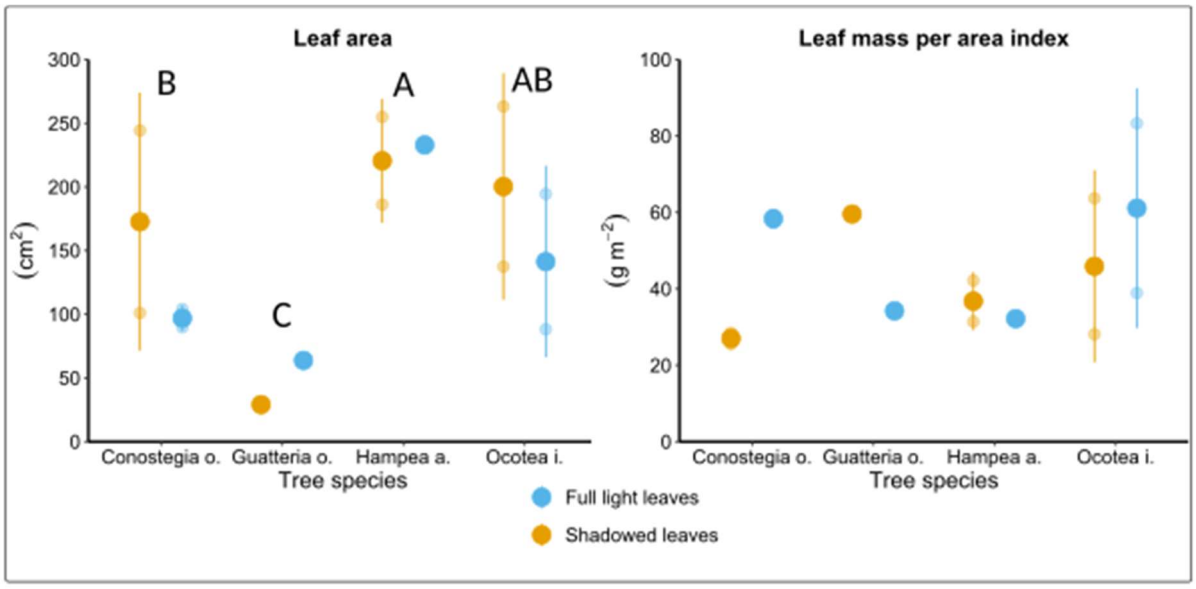

Figure 6: Leaf area (left) and Leaf mass per area index (right) of the sampled trees. Different colors indicate different light exposure, as reported in the legend. Capital letters indicate differences across species, regardless the canopy position (SNK test).

Canopy height and associated differences in light conditions can significantly affect photosynthesis and hence carbon isotope discrimination, which is then reflected in the leaf $\delta^{13}C$ (Farquhar et al. 1989; Ometto et al. 2006; Martin et al. 2020). Our findings indicate that both the tree species and canopy position may potentially influence foliar $\delta^{13}C$ as presented in Table 1. However, the significant effect of canopy exposure varies depending on the tree species. Among all the tested tree species, *Conostegia oerstediana* displayed the highest $\delta^{13}C$ values under light-exposure conditions. Conversely, *Hampea appendiculata* and *Ocotea insularis* exhibited lower foliar $\delta^{13}C$ values, which were associated with relatively high leaf areas (Figure 6). Under shade conditions, the species *Ocotea insularis* maintained the lowest foliar $\delta^{13}C$ values, suggesting a potential disadvantage in terms of WUE compared to the other species. *Guatteria oliviformis* had the lowers leaf area (Figure 6), which could exmplain the more conservative water strategy (higher $\delta^{13}C$ values) compared to *Ocotea insularis* and Hampea *appendiculata*. Indeed, *Guatteria oliviformis* did not exhibit higher $\delta^{13}C$ values in either of the tested canopy exposure positions.

Table 1: Results of the 2-way ANOVA for foliar C/N, N (%), and C and N isotopic composition ($\delta^{13}C$ and $\delta^{15}N$, ‰). The two factors analysed were species (Conostegia o, Hampea a, Guatteria o, and Ocotaea i) and canopy position (sun light or shadowed trees). For each parameter, significant differences ($p \leq 0.05$) are indicated in bold. Capital letters indicate statistical differences within tree species across different canopy light exposure, while lowercase letters denote differences across tree species in the same canopy ligh exposure condition (SNK test).

		C/N		N (%)	
	Canopy position:	*Sun light*	*Shade*	*Sun light*	*Shade*
Species	Conostegia *oerstediana*	21.98 (±1.55) Aa	17.33 (±0.35) Bb	1.78 (±0.11) Bc	2.21 (±0.05) Ab
	Hampea *appendiculata*	12.04 (±0.15) d	11.96 (±0.60) c	3.50 (±0.07) a	3.52 (±0.19) a
	Guatteria *oliviformis*	15.95 (±0.16) Bc	19.10 (±0.03) Aab	2.71 (±0.04) Ab	2.29 (±0.02) Ba
	Ocotea *insularis*	18.64 (±1.10) b	20.26 (±2.13) a	2.48 (±0.12) b	2.28 (±0.22) a
	Two-way ANOVA	p		p	
	Species	**<0.001**		**<0.001**	
	Position	0.986		0.419	
	Species x Position	**<0.001**		**<0.001**	

		$\delta^{13}C$ (‰)		$\delta^{15}N$ (‰)	
	Canopy position:	*Sun light*	*Shade*	*Sun light*	*Shade*
Species	Conostegia *oerstediana*	-30.19 (±0.13) Aa	-34.83 (±0.76) Ba	-0.78 (±0.64)	0.02 (±1.20)
	Hampea *appendiculata*	-36.24 (±0.74) c	-34.90 (±0.17) a	0.68 (±2.23)	0.62 (±0.06)
	Guatteria *oliviformis*	-34.31 (±0.03) b	-35.27 (±0.05) a	-0.47 (±0.01)	0.14 (±0.11)
	Ocotea *insularis*	-36.26 (±0.97) c	-37.40 (±1.06) b	-0.33 (±0.73)	-0.47 (±0.54)
	Two-way ANOVA	p		p	
	Species	**<0.001**		0.375	
	Position	**0.001**		0.515	
	Speciesx Position	**<0.001**		0.825	

4.4.3. Foliar N concentration and isotopic composition

The foliar N concentration observed ranged between 1.7 and 3.7 % (Table 1) and were consistent with foliar N concentration data collected by Nadkarni et al (2004) at the same site. We observed that foliar N concentration strongly depended by the tree species, rather than the tree canopy position. For istance, *Hampea appendiculata* showed higher N concentration in both the canopy light exposure positions, compared to the other tree species. Conversely, *Conostegia oerstediana* showed the lowest foliar N concentration, both in sun-light and in shaded position. Moreover, *Guatteria oliviformis* and *Conostegia oerstediana* showed significant differences in N concentrations

due to the canopy light exposure position, but in opposite directions: while N concentration rised with light exposure in *Guatteria oliviformis*, the opposite was observed in *Conostegia oerstediana*.

Remarkably, we observed a negative correlation between foliar N concentration and $\delta^{13}C$, as illustrated in Figure 7.A. The established positive link between increased foliar N concentration and WUE primarily occurs in N-limited forest ecosystems, where tree growth is restricted by N availability, as seen in temperate forests (Guerrieri et al., 2011). However, this paradigm does not always apply to tropical forest ecosystems, where phosphorus limitation is more prevalent than N limitation (Huang et al., 2016). Additionally, in the intricate structure of a tropical forest with multiple canopy layers, light availability might emerge as a critical limiting factor for growth.

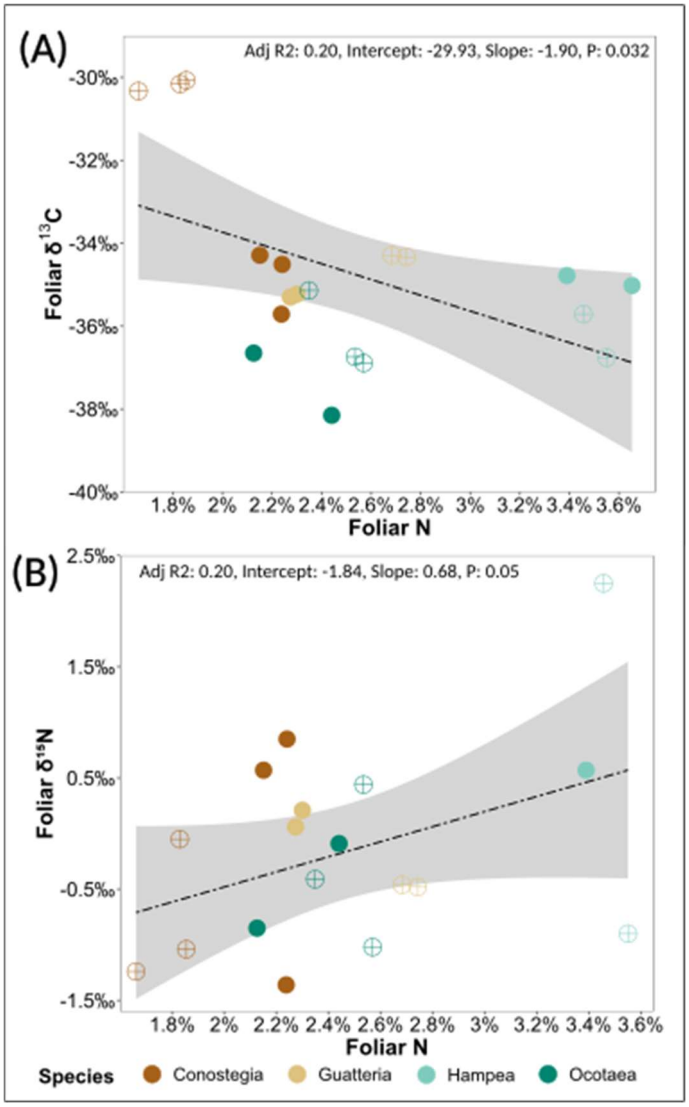

Figure 7: Linear relationships between foliar C isotope composition ($\delta^{13}C$) and foliar N concentration (panel A), and between foliar N isotopic composition ($\delta^{15}N$) and foliar N concentration (panel B). Each point represents a single value referred to the tree units. Different colors indicate the tree species, as reported in the legend. Crossed circles referred to full sunlight trees, while filled circle referred to canopy-shaded trees. The correlation coefficient (Adj R2) and significance level (p) are reported on the top of each panel.

The foliar N isotopic natural abundance ($\delta^{15}N$) exhibited no differences either among various tree species or under different canopy light exposures. Foliar N was enriched in the heavier ^{15}N isotope respect to soil N isotopic composition, with $\delta^{15}N$ values ranging between -1.36 and 2.25‰. Elevated foliar $\delta^{15}N$ values are typically noted in N-rich ecosystems (Craine et al., 2009), and our observations align with this trend, showing an increase in foliar $\delta^{15}N$ values with rising foliar N concentration (Adj R^2: 0.20, p: 0.05, Figure 7.B). Additionally, tropical forests, characterized by N abundance and thriving in warmer climates, generally display higher foliar $\delta^{15}N$ values compared to temperate forests (Martinelli et al., 1999), reflecting the more accelerated N cycling compared to temperate and boreal ecosystems.

4.5. Conclusion

This preliminary research study offers important insights into the C and N dynamics of TMCFs. The observed wide variability in soil C concentration and its negative correlation with $\delta^{13}C$ values, coupled with the positive correlation between soil $\delta^{13}C$ and $\delta^{15}N$, emphasizes the significance of organic soil horizons in shaping the C and N dynamics within TMCFs.

The variability in foliar N concentration was predominantly attributed to tree species rather than light conditions, highlighting the species-specific nature of nutrient dynamics within the studied TMCFs. In contrast, both canopy exposure and tree species were insufficient in explaining the variability in foliar $\delta^{15}N$, suggesting that other factors or interactions may contribute to the N isotopic composition in the foliage. However, the study revealed that both tree species and canopy exposure play pivotal roles in influencing foliar $\delta^{13}C$ values and hence WUE. The variability in foliar $\delta^{13}C$ suggests inter-species differences in water-use strategy. Consequently, the fluctuations in cloud cover and mist dynamics, acting as crucial water sources, could significantly impact the water balances within the forest canopy. This emphasizes the potential vulnerability of TMCFs to environmental changes, particularly alterations in precipitation frequency and cloud/fog formation dynamics.

For future development of this research, it is crucial to integrate repeated monthly sampling campaigns to detect seasonal trends and physiological responses. This should be coupled with punctual meteorological data and measurements of fog/rainfall/tree water $\delta^{18}O$ and air $\delta^{13}C$ to elucidate the role of fogs and rains as plant water source and their role in affecting plant water-use efficiency. This understanding becomes particularly crucial in the context of changing climate conditions and anthropogenic pressures, in order to preserve these unique and ecologically significant ecosystems. In this context, the ongoing collaboration between meteorology, atmospheric physics, and forest ecophysiology has the potential to provide a holistic understanding of the intrinsic relationships between plant physiology, microclimate conditions, and ecosystem processes.

4.6. Acknowledgments

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5. General Conclusion

Research presented in these three articles provides important insights into the responses of forest ecosystems to various anthropogenic pressures.

The application of N manipulative experiments in mature forest ecosystems provided a valuable approach to untangle the effects of N deposition from other global change drivers. This affirmed the significance of maintaining field trials, simulating specific environmental conditions, as a valuable source of data for model validation as well as for improving model predictions. The first scientific article focused on the impact of N addition on temperate beech forests in different climatic and management conditions. Despite the variation observed in nutrient and pigment concentrations, neither of the sites exhibited evident deficiencies or leaf chlorosis. The results suggest that eutrophic beech forests, at least in the medium time period, do not manifest critical nutritional imbalances in response to N addition. However, slight modifications indicative of an incipient stress response were observed, emphasizing the need for continued monitoring and extended investigations to comprehend the long-term effects.

The second article delved into the complexity of using natural N isotope abundances to understand N dynamics within forest ecosystems under enhanced N deposition. While challenges arose in quantifying N retention vs. losses, natural $\delta^{15}N$ emerged as a reliable indicator of forest N cycling. The study underscored the intricate interactions among soil, microbes, plants, and environmental factors influencing N dynamics, offering valuable insights into the impacts of different fertilization approaches and magnitude of doses applied on forest nitrogen cycling.

The third research study explored the C and N dynamics of tropical montane cloud forests (TMCFs). The wide variability in soil carbon concentration, along with species-specific nutrient dynamics, highlighted the importance of organic soil horizons in shaping the C and N dynamics within TMCFs. The variability in leaf $\delta^{13}C$ among the considered species may indicate differences in physiological strategies that can influence adaptations to changes in humidity conditions. The variability in leaf $\delta^{13}C$ among the considered species may indicate differences in physiological strategies that can influence adaptations to changes in humidity conditions. The remarkably low foliar $\delta^{13}C$ values (which is in line with global analyses) underscored the strong dependence of this forest ecosystem on water availability. Hence, the study emphasized the potential vulnerability of TMCFs to environmental changes, particularly alterations in precipitation frequency and cloud/fog formation dynamics, emphasizing the need for continued research to understand these unique ecosystems.

Collectively, these studies contribute to advancing our understanding of the complexities and vulnerabilities of forest ecosystems in the face of global change. Long-term monitoring both under

ambient or controlled conditions (e.g., manipulation experiments) are essential to unravel the intricacies of these responses and inform effective conservation and management strategies.