



# Canopy Exchange and Modification of Nitrogen Fluxes in Forest Ecosystems

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## Abstract

**Purpose of Review** We provide an overview of the main processes occurring during the interactions between atmospheric nitrogen and forest canopies, by bringing together what we have learned in recent decades, identifying knowledge gaps, and how they can be addressed with future research thanks to new technologies and approaches.

**Recent Findings** There is mounting evidence that tree canopies retain a significant percentage of incoming atmospheric nitrogen, a process involving not only foliage, but also branches, microbes, and epiphytes (and their associated micro-environments). A number of studies have demonstrated that some of the retained nitrogen can be assimilated by foliage, but more studies are needed to better quantify its contribution to plant metabolism and how these fluxes vary across different forest types. By merging different approaches (e.g., next-generation sequence analyzes and stable isotopes, particularly oxygen isotope ratios) it is now possible to unveil the highly diverse microbial communities hidden in forest canopies and their ability to process atmospheric nitrogen through processes such as nitrification and nitrogen fixation. Future work should address the contribution of both foliar nitrogen uptake and biological transformations within forest canopies to whole ecosystem nitrogen cycling budgets.

**Summary** Scientists have studied for decades the role of forest canopies in altering nitrogen derived from atmospheric inputs before they reach the forest floor, showing that tree canopies are not just passive filters for precipitation water and dissolved nutrients. We now have the technological capability to go beyond an understanding of tree canopy itself to better elucidate its role as sink or source of nutrients, as well as the epiphytes and microbial communities hidden within them.

**Keywords** Nitrogen fluxes · Nitrogen deposition · Forest canopy · Stable isotopes · Phyllosphere · Epiphytes · Nitrogen retention · Nitrogen assimilation · Nitrification · Nitrogen fixation · Soil canopy · Microbes

## Introduction

Earth's climate is significantly affected by the continuous exchange of water, carbon dioxide (CO<sub>2</sub>), and energy between forests and the atmosphere. Tree canopies remove about 30% of the atmospheric dioxide (CO<sub>2</sub>) emitted by anthropogenic activities through photosynthesis. This removal contributes to the terrestrial CO<sub>2</sub> sink, which has doubled over the last

decade compared to the 1960s, from 1.3±0.4 Gigaton (Gt) C year<sup>-1</sup> to 3.4 ± 0.9 Gt C year<sup>-1</sup> during 2009–2018 [1]. Through stomata on foliar surfaces, water moves from the soil to the atmosphere during transpiration, a process intrinsically associated with CO<sub>2</sub> uptake and that plays a key role in hydrological cycling. It is estimated that about 40% of incident precipitation around the globe is returned back to the atmosphere through transpiration by forest canopies [2]. Transpiration through tree canopies can feedback to climate by cooling the air through latent heat flux or through contributions to cloud formation and reductions in incoming solar radiation. However, forest canopies can also lead to warming through their relatively low albedo and release of water vapor, a potent greenhouse gas [3].

Water and carbon dioxide, however, are not the only compounds that tree canopies exchange with the atmosphere. Indeed, canopies are continuously exposed to chemical compounds emitted into the atmosphere by natural and

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anthropogenic activities and deposited back to the biosphere as wet and dry deposition (ref. chapter 2). Among them, nitrogen in atmospheric deposition has received a lot of attention over the last several decades because of its role in affecting carbon, nitrogen, and water cycling. A positive relationship exists between rates of nitrogen deposition and leaf [4–6••] or canopy [7] photosynthesis, tree growth [8, 9], and water-use efficiency [10–12], due to nitrogen availability strongly affecting all of these processes. Elevated rates of nitrogen deposition has been recognized as one of the drivers contributing to enhanced forest carbon sequestration (and hence a form of climate change mitigation), particularly for nitrogen limited ecosystems in temperate and boreal biomes [13–17]. Greater nitrogen content in tree canopies due to an increase in atmospheric nitrogen input can also feedback to climate by increasing albedo ([18] - positive feedback) and evapotranspiration ([19] - negative feedback).

Increases in atmospheric nitrogen deposition can also accelerate rates of nitrogen cycling, leading to a cascade of effects [20] that eventually shifts the balance between accumulation to nitrogen loss pathways, increases in eutrophication of nearby waterways, loss of diversity [21–23], and nutrient imbalances, such as phosphorus and cations [24]. The influence of tree canopies in nitrogen cycling has been often assessed in terms of litter production and decomposition, the latter playing a crucial role in soil biogeochemical processes [25]. Yet, tree canopies—and the overlooked life hidden within them (e.g., epiphytes and microbial communities)—can retain, assimilate, and process atmospheric nitrogen, thus changing the quantity (in terms of fluxes) and quality (in terms of nitrogen forms) of atmospheric nitrogen inputs eventually reaching the forest floor and soils beneath. However, the mechanisms underlying these differences between what goes into, through, and out of forest canopies are still debated.

Here, we provide a comprehensive overview of how tree canopies contribute to nitrogen cycling, by bringing together knowledge scientists have learned over the last several decades from a variety of research and methodological approaches (i.e., manipulation experiments vs. observations along environmental gradients), and scales, going from the whole ecosystem (e.g., quantification of forest nitrogen fluxes) to canopies (e.g., investigation of foliar nitrogen uptake) down to genes (with omic techniques, and gene-specific quantitative PCR; qPCR). We begin by providing a general introduction to atmospheric nitrogen deposition, focusing mostly on inorganic nitrogen. We then describe what happens when atmospheric nitrogen interacts with tree canopies and the life hidden within them, elucidating three processes: retention, assimilation, and biological transformation (Fig. 1). Last, we identify knowledge gaps and how they can be addressed with future research.

## Quantifying and Monitoring Atmospheric Nitrogen Deposition

Concentrations of reactive inorganic nitrogen (N) compounds in the atmosphere, i.e., reduced forms ( $\text{NH}_3$ ,  $\text{NH}_4^+$ ) and oxidized forms ( $\text{NO}$ ,  $\text{NO}_2$ ,  $\text{NO}_3^-$ ,  $\text{HNO}_3$ , and  $\text{N}_2\text{O}$ ), have increased by 10-fold since 1860, from 15 to 156 Teragram (Tg) N year<sup>-1</sup> in the early 1990s and to 210 Tg N year<sup>-1</sup>. Asia, North America, and Europe are hotspots for these atmospheric nitrogen inputs [26, 27]. In particular,  $\text{N}_2\text{O}$  emissions have increased by more than 20% (from 270 parts per billion (ppb) in 1750 to 331 ppb in 2018), which has positive feedbacks to climate change since it is a greenhouse gas [28]. Intensive agriculture (including livestock) for food production that has sustained human growth is responsible for about 85% of global  $\text{NH}_3$  emissions [29] and for more than 70% of global  $\text{N}_2\text{O}$  emissions [28]. Galloway and Cowing [20] estimated that only a limited amount of the applied fertilizer is recovered in the produced food, and that 80 and 90% of the supplied nitrogen in agricultural systems to grow plants for food production or to feed livestock for meat production is either recycled in the agroforestry systems or lost to the environment (through  $\text{NO}_3^-$  leaching, denitrification, or  $\text{NH}_3$  volatilization). Emissions from power plants and road transport are the main sources of the increase in oxidized nitrogen compounds to the atmosphere [30]. Some components of reactive nitrogen enter terrestrial and aquatic ecosystems as wet (dissolved in precipitation or fog) and dry (as gaseous processes and particles) deposition.

Regional networks have been established worldwide, with the aim of in-situ monitoring of air quality, including changes in reactive nitrogen concentrations and fluxes. These include the European Monitoring and Evaluation Program (EMEP), the National Atmospheric Deposition Program (NADP), and Clean Air Status and Trends Network (CASTNET) in the USA (with the NADP/National Trends Network (NTN) extending to Canada, and Mexico), the Canadian Air and Precipitation Monitoring Network (CAPMoN) in Canada, the Nationwide Nitrogen Deposition Monitoring Network (NNDMN) operated by China Agricultural University, and the Acid Deposition monitoring network (EANET) in East Asia. In Africa, monitoring of wet and dry deposition started in the late 1990's at 10 sites in the west and central parts of the continent, within the IDAF (International Global Atmospheric Chemistry (IGAC)/Deposition of Biogeochemically Important Trace Species (DEBITS)/Africa), which is still active at 3 sites [31••]. To gain a more detailed spatial understanding of trends in deposition and their effects on natural ecosystems, intensive monitoring networks have been established in forests across Europe, such as the International Co-operative Program on Assessment and Monitoring of Air Pollution Effects on Forests (ICP Forests), which has been active since in 1995.

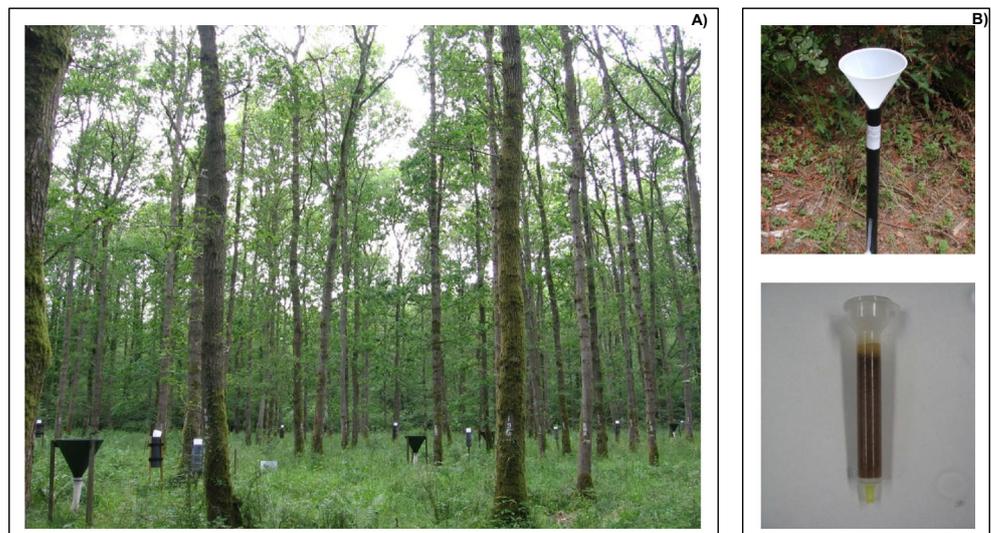
**Fig. 1.** Overview of the main processes underpinning the interactions between tree canopies and atmospheric nitrogen deposition. Numbers refer to processes we describe in the text: 1- retention of nitrogen deposition by tree canopies and epiphytes; 2- foliar nitrogen uptake; 3- leaching of dry nitrogen deposition and dissolved organic nitrogen; 4- microbial nitrogen transformations (e.g., nitrogen fixation, nitrification). Light blue and yellow circles indicate wet and dry nitrogen deposition, respectively. Illustration describing process 1- was modified from Gotsch et al. (2017)



Quantification of inorganic atmospheric nitrogen deposition fluxes is carried out by measuring  $\text{NH}_4^+$  and  $\text{NO}_3^-$  concentrations in water collected in the open areas outside the forest (i.e., wet only or bulk deposition) and beneath tree canopies (i.e., throughfall deposition, Fig. 2A), which are then used in conjunction with measurements of precipitation volume to calculate fluxes. Collectors for bulk and wet deposition are placed in an open space, outside the forest, so to collect the total atmospheric nitrogen deposition (mostly as wet

deposition). However, some of the dry nitrogen compounds can also be deposited in bulk collectors, unless wet-only collectors are installed, which are equipped with a lid that closes during dry periods and only open during precipitation events. These approaches have allowed researchers to document how wet nitrogen deposition has changed over the last decades across Europe and North America [32, 33] and also to identify hotspots such as areas where critical loads have been reached in natural [34] or urban [35•, 36•] ecosystems.

**Fig. 2.** Monitoring of nitrogen deposition in forests by using throughfall water collectors (panel A) and ion exchange resins, IER (panel B). Photo in the panel A refers to an oak forest within the ICP forest network in Europe (i.e., Alice Holt forest in the UK), while in the panel B we show a close-up photo of the ion exchange resin collector. Photos were taken by Rossella Guerrieri (panel A) and Pamela Templer (panel B).



Another approach widely used in the USA (see e.g., [37, 38] – Fig. 2B) and recently in Europe [39•] and China [40], is to incubate mixed ion (i.e. cation plus anion) exchange resins in the field for several weeks at a time. Ion exchange resin (IER) columns can be placed in open areas (outside the forest) to measure bulk deposition or beneath the canopy to measure throughfall, which as in the case of classical water throughfall collectors, provide estimates of wet plus dry deposition since particulates settle on leaves and get washed into collectors when there is precipitation ([41] and reference therein). A number of studies showed that quantification of nitrogen deposition fluxes based on the IER method was comparable to that obtained from traditional water collection [37, 39, 41]. The advantage of using the IER method is that it allows for greater site replication since it requires fewer sampling times compared to the traditional approach that requires weekly collections. However, it has been shown that the IER approach could overestimate  $\text{NH}_4\text{-N}$  deposition, due to the release of  $\text{NH}_4^+$  from the amine compounds from the anion exchange resin polymer, but it could underestimate  $\text{NH}_4\text{-N}$  deposition during heavy rain events. We recommend that readers read relevant studies by Fenn and Poth [37], García-Gomez et al. [39], and Fenn et al. [41] for more details on the limitations of the IER approach, as well as recommendations to improve quantification of inorganic nitrogen deposition with this approach.

One limitation of the estimates from national networks is that they do not provide a clear picture of total nitrogen deposition on a given area, given the difficulties of quantifying reactive dry nitrogen compounds in the atmosphere (and hence their deposition), which has been shown to be an important proportion of total nitrogen deposition [42–45]. Compared to wet N deposition, quantifying dry N deposition is still challenging due to the lack of intensive dry deposition monitoring networks, the difficulties of measuring reactive nitrogen compounds, and the challenge of including deposition processes in models [46]. A very simplistic approach used to estimate dry nitrogen deposition is to calculate the difference in nitrogen fluxes between throughfall and bulk deposition [48•]. Because of their roughness and high turbulence, tree canopies are efficient at absorbing or scavenging reactive nitrogen from the atmosphere [49]. The deposited reactive nitrogen is then washed off during a rain event so that water collected in throughfall reflects both wet and dry reactive nitrogen. This approach, however, has a number of limitations, which will be discussed later (cf. chapters 3 and 5).

A number of networks have been established to monitor gaseous reactive nitrogen, with particular focus on ammonia—see e.g., Ammonia Monitoring Network (AMoN) in the USA (in addition to CASTNET, where gas and particulate air concentrations of  $\text{HNO}_3$ ,  $\text{NH}_4^+$  aerosol, and  $\text{NO}_3^-$  aerosol are included), the Ammonia Monitoring Network in China (AMoN-China), and a number of initiatives in the European Continent (Measuring Ammonia in Nature

network, MAN in the Netherlands or National Ammonia Monitoring Network, NAMN in the UK, and the European Monitoring and Evaluation Program, EMEP, across Europe).

Estimating dry nitrogen deposition is more complex than what we described already for wet deposition and involves measurements and modeling approaches. At the monitoring sites where concentrations of gaseous nitrogen compounds are measured (commonly with passive samplers, filter packs, denuders [41, 47, 50]), dry deposition can be estimated by using inferential modeling approach, which consider the deposition velocities of a given compound in relation to the land-use and the vegetation type, but also canopy conductance [46, 51]. Dry deposition can also be estimated by considering the differences in ion concentrations between bulk vs. throughfall water fluxes in the so-called canopy budget model ([52], ref. chapter 4). A number of studies from the early 2000s have shown the great potential of micrometeorological approaches, such as the eddy covariance technique (EC), to estimate reactive nitrogen fluxes directly (e.g., [53, 54] for  $\text{NH}_3$ , [55] for  $\text{NO}_y$ , [56] for total reactive nitrogen, [45] for a review of its application in the USA). Its applicability, though, is challenged by the very sophisticated instruments required, as well as the high reactivity of dry nitrogen compounds [56].

In-situ monitoring (either via sampling of gaseous concentrations or EC flux measurements) are often spaced apart and are typically located in rural areas to gain regional understanding of atmospheric deposition trends, but still they are limited in number to capture other processes related to transport of dry nitrogen forms [57]. The limited spatial coverage of ground-based dry nitrogen deposition monitoring can be overcome by considering inventory emissions (such as the Emissions Database for Global Atmospheric Research, EDGAR, led by the Joint Research Center in Europe [58]) and atmospheric remote sensing. A number of satellite-based monitoring of surface pollutants have been established (NASA Aura Ozone Monitoring Instrument, ESA Tropospheric Ozone Monitoring Instrument, TROPOMI - see [59] for a review, [60]), representing an important advance to fill the data gap for gaseous nitrogen compounds. Estimates of dry nitrogen deposition at the regional or global scale (either considering ground-based or inventory emissions and satellite-derived nitrogen concentrations) rely on modeling approaches, such as chemical transport models, e.g., EMEP MSC-W [46, 57], LOTUS-EUROS [61], and GEOS-CHEM (<http://acmg.seas.harvard.edu/geos/>). We refer readers to the studies by Theobald et al. [62] for a comparison among different chemical transport models, and by Dentener et al. 2006 [63] and Vet et al. 2014 [64] as example of estimates of atmospheric nitrogen deposition based on an ensemble of chemistry transport models. Since our goal in this chapter is to provide an overview of the approaches at different scales to estimate reactive nitrogen emissions and deposition, we refer readers to Liu et al. [65•] for a review of recent advances about

estimating surface reactive nitrogen concentration and deposition using satellite-based methods and the main challenges associated with these approaches.

Although this literature review mostly focuses on inorganic reactive N, it is important to point out that while monitoring of inorganic nitrogen deposition has intensified over the past decades, this is not the case for organic nitrogen deposition. Measuring concentrations or fluxes of organic nitrogen in deposition is challenging as it is commonly done through the difference between the total nitrogen (i.e. organic + inorganic) and inorganic nitrogen in deposition, which can be labor intensive and expensive [66–68]. The contribution of organic nitrogen forms has been estimated to be on average between 30% and 50% of the total nitrogen deposition based on nitrogen fluxes measurements at 27 locations worldwide [69] or across Europe [66, 67] - with the percentage as high as 70% in the Mediterranean basin [70]. This result suggests that not accounting for organic nitrogen deposition leads to an underestimation of total nitrogen deposition and its contribution to nitrogen cycling [26, 71].

Human activities have increased atmospheric nitrogen inputs to terrestrial ecosystems by 46 Tg N year<sup>-1</sup> compared to pre-industrial time [72], with recent global estimates ranging between 90 [73] and 100 Tg N year<sup>-1</sup> [64, 71], with hotspots in eastern Asia, Europe, eastern North America, and southern Brazil [72]. Of the total N deposition, 70 Tg N year<sup>-1</sup> are deposited onto terrestrial surfaces [27], with about 18 Tg N year<sup>-1</sup> reaching forest ecosystems [72].

Results from long-term monitoring networks reported a significant reduction in nitrogen (particularly the oxidized forms) and sulfur deposition (e.g., [32, 45] in Europe and North America; [33] based on measurements at the European ICP Forests) over the last several decades. Rates of total nitrogen deposition are generally elevated in urban compared to nearby rural areas [74]. However, decreases over time were steeper for sulfur compared to nitrogen deposition, particularly in the case of NH<sub>3</sub> deposition [43, 48]. A recent study by Fenn and colleagues [75] highlighted that on-road emissions of NH<sub>3</sub> have increased in the USA due to the introduction of vehicles equipped with new engine technologies, where aqueous urea is injected as a reductant for NO<sub>x</sub> control, leading to the production of NH<sub>3</sub> [76, 77]. Actions for abatement of NH<sub>3</sub> emissions and hence, deposition are more difficult to put in place, given their link to food production [78]. In contrast to North America and Europe, China has experienced an overall increase in nitrogen deposition over recent decades [79]. Moreover, global estimates from satellite-based observations and modeling highlighted the increase in nitrogen deposition in tropical regions [26, 63, 73], due to the intensification of agriculture (and hence increasing use of synthetic fertilizers) and biomass burning associated with deforestation [80, 81].

## Tree Canopies and Nitrogen Fluxes: What Comes in From the Atmosphere does not Always Reach Soils Beneath

Where does atmospheric nitrogen go when moving through forest ecosystems and how does atmospheric nitrogen deposition affect processes occurring within tree canopies? Three approaches have been used to answer this question: nitrogen manipulation experiments (either on seedlings or on mature trees in forests), observations across sites within long-term nitrogen monitoring networks, and individual observational studies. We summarize all three below.

To follow the fate and activity of atmospherically deposited nitrogen to forest ecosystems, nitrogen is often added in amounts and forms that mimic atmospheric deposition, mostly as ammonium nitrate (NH<sub>4</sub>NO<sub>3</sub>; with labeled <sup>15</sup>N fertilizer in some cases to track specific cohorts of nitrogen throughout an ecosystem). Past studies, mostly considering soil nitrogen fertilizations, showed that soil and not trees retain most of the nitrogen that comes from atmospheric deposition (e.g., Emmett et al. [82], presenting results from the manipulation experiments within NITREX project across European temperate and boreal forests; Nadelhoffer et al. [83], for results on the long-term soil manipulation experiment at Harvard forest in the USA; Gurmessa et al. [84], in a humid tropical forest in China; Schlesinger [72] and Templer et al. [85] for two analyses across many studies). Moreover, from soil fertilization experiments researchers found that the simulated increase in nitrogen deposition had a significant effect on the biogeochemical processes, leading to soil acidification, increases in NO<sub>3</sub><sup>-</sup> leaching and nutrient imbalances [86–89], and shifts in bacterial [90] and/or fungal communities [91].

Tree canopies are critical to our understanding of the nitrogen cycle since they can modify the amount and form of nitrogen entering the forest floor through retention, transformation, and absorption by foliage, a process often excluded in previous soil manipulation experiments. Some past and more recently established studies have mimicked the increase in nitrogen deposition using mist applications directly to tree canopies in forests or at the watershed scale (ref. Table 1) to investigate the effects of increasing nitrogen deposition on nitrogen cycling at the whole ecosystem scale. Among those experiments, however, only a few have specifically elucidated the interactions between atmospheric nitrogen deposition and tree canopies, whose results will be discussed in this chapter. The first canopy nitrogen addition experiment we are aware of was carried out on a young Sitka spruce plantation at Deepsyke in Scotland (UK). The experiment included nitrogen misting over tree canopies alone or in combination with sulfur as single or double dose (48

**Table 1** Overview of the experimental sites where nitrogen applications to forest canopies have been carried out. We also include experiments that are no longer active and describe when experimental treatments also include nitrogen applications to soil and the level of ambient atmospheric nitrogen deposition ( $N_{\text{dep}}$ ). CN and CNS indicates canopy nitrogen and canopy nitrogen + sulfur aerial misting, respectively,

whereas SN indicates soil nitrogen applications. Numbers following CN, CNS or SN indicate that different doses were applied, for a given treatment. (\*) Values were calculated as half of the dose applied, as Adams et al. (2007) reported additions of N and S are approximately twice the ambient nitrogen deposition in the adjacent watershed considered as control

Name of the site (Region, Country)	Forest type (dominant tree species)	Forest age	Ambient $N_{\text{dep}}$ ( $\text{kg ha}^{-1} \text{year}^{-1}$ )	N dose applied ( $\text{kg ha}^{-1} \text{year}^{-1}$ )	Duration	Reference
Deepsyke (Scotland, UK)	Conifer forest ( <i>Picea sitchensis</i> (Bong.) Carr.)	30	8-10	CN: 48 CNS1: 48 CNS2: 96	1996-2003	Sheppard et al. 2004; [92] Guerrieri et al. 2011 [10]
Howland forest (Maine, USA)	Conifer forest ( <i>Picea rubens</i> Sarg., <i>Tsuga canadensis</i> (L.) Carr.)	~140	< 5	18	2001-2005	Gaige et al. 2007; Dail et al. 2009 [93, 94]
Cansiglio (Veneto, Italy)	Deciduous forest ( <i>Fagus sylvatica</i> L.)	~140	20	CN: 30 SN1: 30 SN2: 60	2015-present	-
Cembra (Trentino Alto Adige, Italy)	Deciduous forest ( <i>Fagus sylvatica</i> L.)	60	< 10	CN: 20	2018-present	-
Monticolo (Trentino Alto Adige, Italy)	Deciduous forest ( <i>Quercus petraea</i> L.)	67	6.6	CN: 20	2015-present	Giammarchi et al. 2020 [95]
Jigongshan National Natural Reserve (Henan, China)	Mixed deciduous forest ( <i>Quercus acutissima</i> Carruth, <i>Quercus variabilis</i> Bl., <i>Liquidambar formosana</i> Hance)	45	19.6	CN1: 25 CN2: 50	2013-present	Zhang et al. 2015 [96•]
Shimentai National Natural Reserve (Guangdong, China)	Broadleaved evergreen forest ( <i>Cryptocarya concinna</i> , <i>Schima superba</i> , <i>Machilus chinensis</i> , <i>Castanea henryi</i> (Skan) Rehd, <i>Engelhardtia roxburghiana</i> )	50	34.1	SN1: 25 SN2: 50	2013-present	Zhang et al. 2015 [96•]
Bear Brook (Eastern Maine, USA)	Mixed deciduous forest with some conifers at high elevation	50-80	8.4	CN: 25.2 CNS: 28.8	1989-2016	Fernandez et al. 2003 [97]
Fernow (West Virginia, USA)	Mixed deciduous forest	34-95	17.5*	CN: 35.5 CNS: 40.5	1989-	Adams et al. 2007 [98]
Monts-Valin and Simoncouche (Quebec, Canada)	Conifer forest ( <i>Picea mariana</i> (Mill.) BSP)	80	0.7-1	CN: 2	2008-2016	De Barba et al. 2016 [99]
Prades (Cataluña, Spain)	Evergreen forest ( <i>Quercus ilex</i> L.)	80	15	CN: 60	2015-present	-

and  $96 \text{ kg ha}^{-1} \text{ year}^{-1}$  for both nitrogen and sulfur) for 5–8 years so to simulate an increase in acid deposition (cf. [92, 100]). Another remarkable manipulation experiment was carried out at Howland in Maine (USA), where an increase in nitrogen deposition over a mature spruce-fir forest was simulated by water drop through a helicopter for 5 years (Table 1). Both experiments showed that between 20–40% (at Deepsyke Forest [101, 102]) and up to 70% (at Howland Forest [93]) of the applied nitrogen remained in tree canopies. A similar percentage of nitrogen canopy retention (30–50%) was reported in a recently established (and still active) manipulation experiment in

an evergreen broad-leaved forest [6] and mixed deciduous forest [103] in China at the two forest sites described in Table 1. Different dynamics of canopy nitrogen exchange were observed at Deepsyke Forest according to whether the nitrogen addition also contained sulfur. In fact, the absolute amount of nitrogen retained as  $\text{NH}_4^+$  and  $\text{NO}_3^-$  was similar regardless of the applied doses (i.e., 40% for both), whereas tree canopies in the nitrogen addition treatment retained more  $\text{NH}_4^+$  (i.e., 60%) than  $\text{NO}_3^-$  (i.e., 8%) [102]. The preferential higher retention of  $\text{NH}_4^+$  vs.  $\text{NO}_3^-$  by tree canopies was also found at Howland Forest. Moreover, at Howland, by carrying out a  $^{15}\text{N}$  tracer

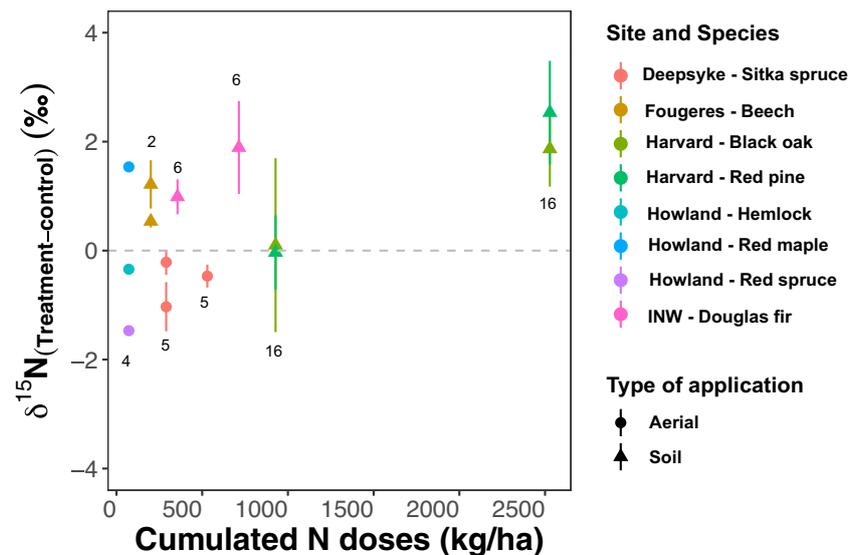
experiment (i.e., spraying trace amounts of enriched  $^{15}\text{N}$ - $\text{NH}_4$  and  $^{15}\text{N}$ - $\text{NO}_3$  on subplots) researchers tracked the fate of nitrogen, including foliage, wood, and bark. Surprisingly, the highest  $^{15}\text{N}$  retention was observed in branches and bark and not in foliage, as expected. This result suggests that the high retention within the ecosystem was more related to physico-chemical interactions between atmospheric nitrogen and plant surfaces rather than direct uptake through foliage [94••]. However, another important result from the manipulation experiment at Howland forest was the rapid conversion of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  into soluble organic nitrogen in tree canopies, which led to an increase in below-canopy dissolved organic nitrogen fluxes [93]. This result was explained by the authors as rapid assimilation of inorganic nitrogen by plants and associated epiphytes, and consequent release as plant, lichen, and microbial exudates, via physical damage to foliage, or via insect mediated release [93]. An increase in organic nitrogen fluxes below-canopies was also detected at Deepseyke Forest but its contribution to total nitrogen fluxes was smaller compared to what was observed at Howland forest, indicating a limited capacity to generate organic nitrogen within the canopy for this ecosystem [104].

A comparison of tree-ring stable nitrogen isotopic composition ( $\delta^{15}\text{N}$  – ref. Appendix 1) (evaluated as difference between nitrogen addition treatment and control—no  $^{15}\text{N}$ -labeling was applied—Fig. 3, modified from Guerrieri et al. [10]) between canopy and soil nitrogen fertilization experiments showed a different pattern between the two approaches considered in terms of ecosystem nitrogen dynamics. With increasing nitrogen dose applied to the soil, the natural abundance  $\delta^{15}\text{N}$  values in tree rings became more  $^{15}\text{N}$ -enriched compared with the control plots, indicating an increase in the coupling of soil nitrogen availability and nitrogen loss pathways (e.g., [105]). The opposite pattern was observed in the case of aerial misting over tree canopies, with a decrease in tree ring natural abundance  $\delta^{15}\text{N}$  values in the nitrogen addition treatment compared to the control, suggesting a higher amount of nitrogen retention in the ecosystem instead [10]. The substantially higher level of canopy (including branches and bark) nitrogen retention in experiments where nitrogen is added to the canopy compared to nitrogen fertilizers to soils [85] shows that forest canopies can contribute significantly to nitrogen immobilization by trees and microbial communities within forest ecosystems and to reducing nitrogen loss pathways. These results also suggest that soil fertilization experiments can overestimate the role of nitrogen deposition in soil biogeochemical processes and overlook the role of tree canopies in retaining atmospheric nitrogen. However, the overall short duration of the manipulation experiments, the high nitrogen doses applied

(compared to the ambient nitrogen deposition), and the difference in terms of forest structure (forest age and management) make it challenging to draw conclusions about long-term responses of canopy vs. soil nitrogen processes to simulated increase in nitrogen deposition.

Monitoring of the specific inorganic nitrogen chemical species (i.e.,  $\text{NH}_4^+$ ,  $\text{NO}_3^-$ ) in deposition and water collected underneath tree canopies as throughfall has shown that nitrogen deposition is substantially altered in its path through the canopy. Hence, comparisons of throughfall to bulk nitrogen deposition can demonstrate whether canopies are net sinks or sources for nitrogen. Increases in nitrogen fluxes in throughfall relative to bulk deposition have been observed particularly at high nitrogen deposition sites, which were attributed to leaching and washing of dry nitrogen deposition from the canopies by precipitation [48•, 66, 106–109]. In this case, tree canopies can still retain part of the atmospheric nitrogen, though this process can be masked by the high wash-off of dry nitrogen deposition [110, 111]. On the other hand, lower nitrogen fluxes in throughfall than bulk deposition have also been reported (in temperate, boreal and tropical forests), and have been considered an indication of retention and consumption by tree canopies and associated epiphytes [4, 110, 112–118] and of direct foliar uptake (e.g. [119, 120] ref. chapter 4). Differences were reported between conifer and deciduous forests in the percentage of canopy retention, with the former showing a higher interception of inorganic nitrogen from precipitation compared to the latter [66, 121]. When focusing on canopy ‘preference’ in terms of nitrogen forms ( $\text{NH}_4^+$ ,  $\text{NO}_3^-$  and dissolved organic nitrogen), interesting differences were found. In general, a higher efficiency by tree canopies in retaining  $\text{NH}_4^+$  rather than  $\text{NO}_3^-$  was reported [113, 117••, 122•]. However, some studies reported a stronger preferential retention of  $\text{NO}_3^-$  compared to  $\text{NH}_4^+$ , particularly under low nitrogen deposition (Fenn et al. [110] and reference therein, particularly Table 4).

The magnitude of canopy nitrogen retention has been associated with both forest nitrogen status and changes in atmospheric nitrogen deposition, with higher retention at low nitrogen availability and under low atmospheric nitrogen input (e.g., [123], [121] across EU, [117••] in Canada). This observation is important, as it suggests that—particularly for nitrogen deposition in the form of  $\text{NO}_3^-$ —canopy retention can significantly reduce the amount of atmospheric  $\text{NO}_3^-$  inputs to the soil (between 30 and 60% of the bulk deposition [66, 117••]) and limit soil nitrogen loss pathways through leaching of  $\text{NO}_3^-$  and gas loss [124, 125]. Different attributes related to forest structure and dynamics (e.g., diversity, phenology) seem also to be relevant in explaining differences in canopy nitrogen retention. A number of studies found that canopy



**Fig. 3.** Results of the meta-analysis presented in Guerrieri et al. (2011), showing natural abundance  $\delta^{15}\text{N}$  values in tree rings (as difference between nitrogen fertilization and control) vs. cumulated doses of nitrogen (N) applied over the duration of the experiments, which is indicated with numbers close to symbols. We updated the original figure in Guerrieri et al. (2011) by including also natural abundance

$\delta^{15}\text{N}$  values measured for red maple, red spruce, and hemlock at Howland forest. Values are means with 95% confidence intervals and were measured during the years fertilization treatments were carried out. See Guerrieri et al. (2011) for more details on the meta analysis and supplementary information for a description of wood core collection and sample preparation at Howland forest.

retention was typically higher during the growing season [121, 126, 127] and that it was associated with forest diversity [121]. The latter is not surprising, particularly if we consider diversity not only in terms of different tree species but also their associated leaf and morphologies traits (which can affect ability of canopies to ‘capture’ atmospheric deposition) and life they host (ref. chapter 6).

Several studies have shown that when water from precipitation cascades through the canopy—via throughfall and stemflow—concentrations of dissolved organic matter (see review by Van Stan and Stubbins, 2018 [128]) and particulate organic matter (organic material ranging from 0.45  $\mu\text{m}$  and 500  $\mu\text{m}$ ; see review by Ward et al. [129]) can be significantly altered. With particular reference to nitrogen, monitoring dissolved and particulate organic nitrogen in bulk deposition and throughfall is not carried out routinely as in the case of dissolved inorganic nitrogen. Hence, it is hard to provide an overall picture about whether and in which direction organic nitrogen fluxes change when passing through tree canopies. Results in the literature vary from no changes [70, 130], to reduction (in tropical and boreal forests [115] and reference therein), and to an increase in dissolved [48•, 70] and particulate [131] organic nitrogen fluxes in throughfall compared to bulk deposition. Causes for the significant increase in both dissolved and particulate organic nitrogen fluxes beneath tree canopies were related to leaching of canopy

herbivore frass [132] or microbial biomass [131, 133••], pollen and bud burst [68•], or caterpillar [134] or fungi [135] infestations. These results suggest that canopy phenology can also control the inputs of organic nitrogen to the soil. Moreover, a more intensive monitoring of organic nitrogen fluxes in forests could help in detecting insect pests, which are becoming more frequent and severe with climate change [66, 136].

### Canopy Nitrogen Retention and Uptake: Why Distinguishing Between the Two Matters

Canopy retention is often referred to as canopy uptake, though the two processes are quite different. Nitrogen that is retained in tree canopies—defined as ‘the atmospheric N input to the canopy not reaching the forest soil’ [126]—could be adsorbed by leaf and branch/bark surfaces and/or associated microbes, but it does not necessarily mean that it is assimilated by plants via their foliage. In this chapter we specifically focus on the foliage nitrogen uptake pathway. Atmospheric nitrogen inputs represent an additional and readily available source of nitrogen that can be actively taken up by foliage and thus, be metabolized by plants [4, 119, 137••, 138•, 139••, 140••, 141]. This pathway can be particularly important for those ecosystems relying on fog for water and nutrients it carries, e.g.,

*Sequoia sempervirens* forests in California [137••] and *Arthroerua leubnitziae* in the Namibia desert [142•], as well as several tree species in wet tropical forest in the Republic of Panama [143••].

How does nitrogen uptake through canopy work and how can it be described? We remind readers of reviews by Sparks [119], Krupa [144] – with focus on  $\text{NH}_3$ , and Hu et al. [145], which provide a comprehensive description of the foliar nitrogen uptake pathways and subsequent transport and metabolism (including associated genes and enzymes). Briefly, there are two pathways for  $\text{NH}_3$  to enter the leaves: through stomata and cuticles ([119, 120] and reference therein). Stomatal uptake of  $\text{NH}_3$  depends on stomatal conductance and on the concentration gradient between atmosphere and substomatal cavities where  $\text{NH}_3$  is dissolved in the water film of the mesophyll cells to form  $\text{NH}_4^+$ . Atmospheric  $\text{NH}_3$  can also passively be deposited on leaf cuticles, where the presence of relative humidity can favor its solubility and conversion to  $\text{NH}_4^+$ , which can then be assimilated via cuticle or be reemitted. Oxidized forms of nitrogen ( $\text{NO}$  and  $\text{NO}_2$ ) enter foliage through stomata and dissolve in the leaf apoplast to primarily form nitrous acid ( $\text{HNO}_2$ ) and nitric acid ( $\text{HNO}_3$ ) that then dissociate to  $\text{NO}_2^-$  and  $\text{NO}_3^-$  [119]. Though here we focus mostly on inorganic nitrogen, it is worth mentioning that foliar uptake of organic nitrogen has also been reported [146, 147].

Nair et al. [139••] conducted a mesocosm manipulation experiment with Sitka spruce saplings where, among other treatments, a solution containing isotopically enriched  $^{15}\text{NH}_4\text{NO}_3$  was sprayed on the soil and applied directly to twigs and needles through a brush presoaked with treatment solution. The input of nitrogen did not significantly add to the ambient nitrogen deposition experienced by the saplings ( $14.5 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ , Nair 2021, personal communication), as only  $54 \text{ g } ^{15}\text{N ha}^{-1} \text{ year}^{-1}$  was added as tracer with the experiment. The study showed that 60% of  $^{15}\text{N}$  applied to the canopy was recovered in the aboveground components of trees (needles, stem and branches), while only 21% was recovered in aboveground biomass when  $^{15}\text{N}$  was applied to the soils. In another experiment carried out by Adriaenssens et al. [126], oak, birch, Scots pine, and beech saplings were exposed to different amounts of labeled  $^{15}\text{NH}_3$ . The authors found greater  $^{15}\text{NH}_3$  uptake by birch, beech, and oak compared to pine, which was attributed to the lower nitrogen requirement by conifers in general, owing to high internal nitrogen recycling and longer retention time for needles [94••]. Moreover, results pointed to a reduction in  $^{15}\text{NH}_3$  uptake at higher dose of nitrogen addition, which was attributed to possible attenuation by surface nitrifying bacteria (ref. chapter 6), increasing temporal storage of  $\text{NH}_3$  or reemission of  $\text{NH}_3$ . In the manipulation experiments carried out at Howland Forest

(ref. chapter 2) differences were observed between species in terms of nitrogen uptake in the subplots where labeled  $^{15}\text{NH}_4^+$  and  $^{15}\text{NO}_3^-$  were sprayed over tree canopies. We already mentioned that contrary to the expectation, retention of the applied nitrogen was observed in branches and bark (particularly in the case of hemlock) rather than foliage. However, when looking at the difference between  $^{15}\text{N}$  values measured in foliage of trees treated with trace amounts of  $^{15}\text{N-NH}_4^+$  vs.  $^{15}\text{N-NO}_3^-$ , results suggested a preferential uptake of  $^{15}\text{NO}_3^-$  compared  $^{15}\text{NH}_4^+$  in the case of hemlock, red spruce, and red maple, whereas the opposite was found for white cedar and white pine. In a manipulation experiment where hybrid poplar seedlings were exposed to  $\text{NO}_2$  fumigation, Siegwolf et al. [148] demonstrated the occurrence of foliar nitrogen uptake and assimilation, the latter confirmed also by an increase in nitrate reductase activity. Similar results were also reported in a subtropical forest in Shimentai National Nature Reserve (China, ref. Table 1), where 2 years of exposure to nitrogen misting over tree canopies resulted in an increase in leaf nitrogen and enzymes associated with leaf nitrogen metabolism (i.e., nitrate and nitrite reductase), though the latter result was not consistent across the three dominant species [6, 149••].

Taken all together, results from these studies suggest that there are differences among species in the primary form of inorganic nitrogen taken up, and that nitrogen uptake could be controlled by the level of nitrogen deposition and by tree age. In this latter case it should be mentioned that only in the case of Howland and Shimentai forests adult trees were considered, whereas most studies focus on saplings [120, 126, 139••], often considering only a single pulse  $^{15}\text{N}$  tracer treatment [141]. The primary study of saplings is not surprising, given that manipulation experiments on small plants are easier to manage and responses to experimental treatment can be detected more quickly than under gradual increase in ambient nitrogen deposition with taller statured mature trees. However, as already highlighted, while manipulation experiments have greatly contributed to our understanding of important processes, such as foliar nitrogen uptake, their results can be biased by the short duration of the experiment and results may be difficult to generalize beyond sites or species considered.

Natural abundance  $\delta^{15}\text{N}$  values in foliage and tree-rings has been used extensively as a tool to assess the assimilation by tree canopies of atmospheric nitrogen, particularly near emission sources such as motorways or industrial activity [150–153]. For example, Ammann et al. [150] observed an increase in  $\delta^{15}\text{N}$  values measured in spruce needles and soil moving from trees near the highway (exposed to pollution) to those 1 km away. The authors attributed this variation in  $\delta^{15}\text{N}$  values to the

decreasing influence of  $\text{NO}_2$  input from traffic. The limitation of this approach, however, is that the isotopic signature of the atmospheric nitrogen sources is not always known, or it relies on point measurements (both in space and time), which makes difficult the interpretation of the long-term isotopic signals detected in plants (see [153, 154] for more details). In situ assessment of foliar nitrogen uptake, particularly in the oxidized form, has recently been assessed using dual ( $\delta^{15}\text{N}$  and  $\delta^{18}\text{O}$ , [155] for a review) or triple ( $\delta^{15}\text{N}$ ,  $\delta^{18}\text{O}$  and  $\delta^{17}\text{O}$ ) isotope approaches. The simultaneous measurement of nitrogen and oxygen isotopes of plant tissue  $\text{NO}_3^-$  has been successfully used to demonstrate foliar  $\text{NO}_3^-$  uptake in mosses [156] and herbaceous species [140••] and to assess plant  $\text{NO}_3^-$  sources and uptake for dominant plant species in Alaskan tundra ecosystems [157••]. Bourgeois et al. [140••] demonstrated not only the foliar uptake of  $\text{NO}_3^-$  in subalpine grassland species, but also seasonal shifts in  $\text{NO}_3^-$  uptake; the plants relied on roots for nitrogen uptake in spring after snowmelt, and on leaves in summer, likely due to higher competition with soil microbes. More studies are needed to confirm whether these seasonal dynamics also occurs with trees.

The canopy nitrogen uptake pathway is included in mechanistic models, such as the canopy budget model [52], which is used to estimate dry deposition. This model is based on balancing ion exchange between canopies and the solutions passing through them, so that the total deposition beneath tree canopies (including throughfall, TF and stemflow, SF) is given by:  $TF + SF = TD + CE = PD + DD + CE$ , where TD and CE are total atmospheric deposition (e.g., open field wet, PD + dry, DD, depositions) and canopy exchange, respectively. The canopy exchange (CE) in the interface between wet atmospheric deposition and tree canopy surface is the main process governing the uptake and release of the major ions. For a given ion, a positive CE value indicates that tree canopies contribute to TF +SF flux via leaching, while a negative CE value is associated with canopy uptake. For inorganic nitrogen, the assumption is that uptake of  $\text{NH}_4^+$  and/or  $\text{H}^+$  by tree canopies goes along with the release of base cations, such as  $\text{K}^+$ ,  $\text{Ca}^{2+}$ , and  $\text{Mg}^{2+}$ . For  $\text{NO}_3^-$  it is often assumed that neither canopy leaching nor uptake occurs ([52] and references therein), though recent developments of the canopy budget model include canopy  $\text{NO}_3^-$  uptake as a proportion of  $\text{NH}_4^+$  uptake [158]. It should be pointed out, however, that the negative CE for  $\text{NH}_4^+$  or  $\text{NO}_3^-$  might indicate retention rather than uptake, as we do not know whether nitrogen is actually assimilated by foliage. Moreover, the model does not account for possible nitrogen transformations occurring in tree canopies by

epiphytes and/or microbes associated with foliage (as we will see in the next chapter). Nevertheless, the approach certainly contributes to improving quantification of total nitrogen deposition reaching forest ecosystems, when direct measurements of dry deposition are not available [48•].

### Biological Transformation in Tree Canopies: Hints From Stable Isotopes of Nitrogen and Oxygen

Tree canopies and epiphytes they host contribute significantly to altering the nitrogen fluxes and type of nitrogen compounds reaching the soil. Nitrogen retention and uptake are the main mechanisms proposed to explain differences in nitrogen fluxes between bulk deposition and throughfall (e.g., canopy budget model). Yet, looking at differences between fluxes together with changes in isotopic composition of different nitrogen forms that enters canopies from the atmosphere and those that pass to the forest floor provide evidence of within-canopy biological transformations. The measure of natural abundance stable isotopes of nitrogen ( $^{15}\text{N}/^{14}\text{N}$ ) and oxygen ( $^{17}\text{O}/^{16}\text{O}$ ,  $^{18}\text{O}/^{16}\text{O}$ ) in forest water has proven to be a powerful tool to characterize the sources of atmospheric nitrogen ([159–161], and [162••] for a review) and to trace its transformations when interacting with tree canopies [111, 163–166••]. We have a comprehensive theoretical understanding of isotopic fractionation during soil biogeochemical processes and nitrogen transfer to plant [167]. Yet, discriminating between nitrogen compounds derived from atmospheric transformations occurring within tree canopies and how they affect the isotopic signatures of N compounds produced (e.g., those taken up and/or leached) are less understood.

For instance,  $\text{NH}_4^+$  and  $\text{NO}_3^-$  derived from dry nitrogen deposition is in general more enriched in  $^{15}\text{N}$  (that is more positive  $\delta^{15}\text{N}$  values) compared to those measured in bulk precipitation [167]. However, a decrease in  $\delta^{15}\text{N}$  in  $\text{NO}_3^-$  from bulk deposition to throughfall was reported in a spruce forest in Germany [168], a montane rainforest in Ecuador [169], and Scots pine in the UK [165••], indicating isotopic fractionation during nitrification of  $\text{NH}_4^+$  to  $\text{NO}_3^-$  in the canopy foliage. Indeed, nitrification of  $\text{NH}_4^+$  leads to the production of  $^{15}\text{N}$ -depleted  $\text{NO}_3^-$  and leaves behind more  $^{15}\text{N}$  enriched  $\text{NH}_4^+$  [167, 170].

More direct evidence of nitrification occurring in tree canopies could derive from the dual isotope approach, which includes measurement of both  $\delta^{15}\text{N}$  and  $\delta^{18}\text{O}$  in  $\text{NO}_3^-$ . This approach has been extensively applied to

investigate soil biological transformations and to assess the nitrogen saturation status of forested catchments by looking at the changes in  $\delta^{18}\text{O}$  of  $\text{NO}_3^-$  in bulk deposition and streamwater [160, 171–173••]. A large difference between the isotopic signature of the atmospherically-derived  $\text{NO}_3^-$  (20 to 80 ‰ – ref. Appendix 1) and the terrestrial  $\text{NO}_3^-$  (particularly biological transformation, such as nitrification) (–10 to +10 ‰, ref. [160] for a review) can help distinguish between the two sources. For instance, based on this approach, a number of studies in the northeastern US [111, 171] found that  $\text{NO}_3^-$  in streamwater comes primarily from nitrification within the catchment rather than directly from atmospheric deposition. Yet, only a limited number of studies used the dual isotope approach to assess the occurrence of canopy nitrification ([111, 165••, 174••]). Increases in  $\text{NO}_3^-$  concentrations and reduction in both  $\delta^{18}\text{O}$  and  $\delta^{15}\text{N}$  in  $\text{NO}_3^-$  in filtered throughfall water collected underneath *Cryptomeria japonica* was attributed to nitrification in tree canopies [174••]. Similar results were reported by Guerrieri et al. [165••] in a beech and Scots pine forests exposed to  $>10 \text{ kg N ha}^{-1} \text{ yr}^{-1}$  in the UK, which was further supported by the isotopic mass balance approach combining the  $\delta^{18}\text{O}$  of the two different sources— atmosphere (wet atmospheric  $\text{NO}_3^-$ ) and nitrification ( $\text{NO}_3^-$  washed off tree canopies and collected in throughfall water). There are, however, limitations of the dual isotope approach (at least when applied to  $\text{NO}_3^-$  in the soil). These include *i*) the fact that there is overlap in isotopic composition among nitrate sources (e.g., nitrification and atmospheric deposition) and *ii*) that the isotopic signature of  $\text{NO}_3^-$  is significantly altered by isotopic fractionation during denitrification—a process that leads to a high enrichment in  $^{15}\text{N}$ - $\text{NO}_3^-$  left behind during the biological process and then available for plant uptake ([160] and references therein, [167], and the earlier study by Mariotti [175]). Moreover, there are uncertainties in the estimates of atmospheric vs. biological  $\text{NO}_3^-$  fractions as obtained by the isotopic mass balance approach, mostly associated with the  $\delta^{18}\text{O}$  produced during nitrification (we refer readers to the discussion in Riha et al. [176] and Guerrieri et al. [165••] for more details).

A more robust approach to partitioning sources of  $\text{NO}_3^-$  is based on measurements of both  $\delta^{17}\text{O}$  and  $\delta^{18}\text{O}$  in  $\text{NO}_3^-$ , which allows researchers to distinguish between atmospheric and microbial (through nitrification) sources of  $\text{NO}_3^-$ . The approach was proposed by Michalski et al. [177, 178••] and since then has been used in a number of studies assessing the source of  $\text{NO}_3^-$  in soil solution in forests [179] or streamwater at the catchment level [176, 180, 181••]. Briefly, mass-dependent isotope fractionation

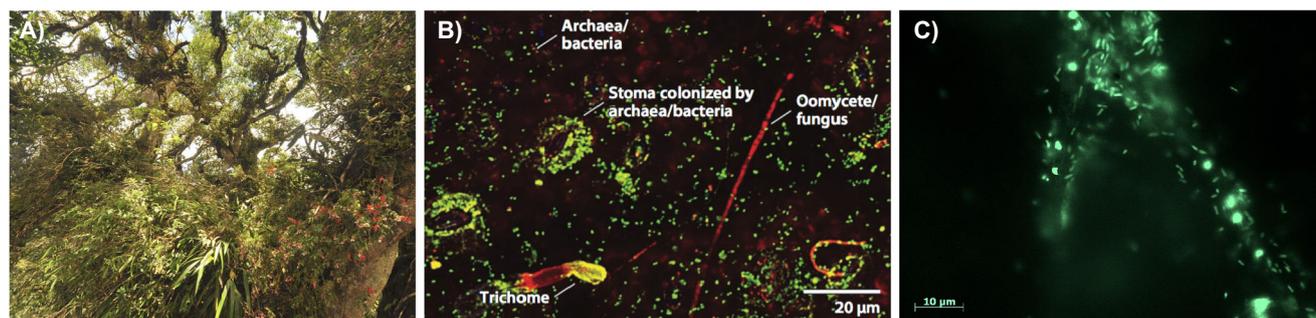
leads to a consistent relationship between  $\delta^{17}\text{O}$  and  $\delta^{18}\text{O}$ , i.e.,  $\delta^{17}\text{O} \approx 0.52 \times \delta^{18}\text{O}$ , which follows the so-called mass dependent fractionation line. However, mass independent fractionation occurs during the formation of ozone and leads to excess in  $^{17}\text{O}$ , which is then reflected in the  $\delta^{17}\text{O}$  of the atmospheric  $\text{NO}_3^-$ . This excess  $^{17}\text{O}$  is quantified by  $\Delta^{17}\text{O} = \delta^{17}\text{O} - 0.52 \times \delta^{18}\text{O}$  [177]. This means that in the case of  $\text{O}_3$ -derived  $\text{NO}_3^-$ ,  $\delta^{17}\text{O}$  values tends to deviate from the mass dependent fractionation line, resulting in  $\Delta^{17}\text{O} > 0$ , while mass-dependent nitrification produces  $\text{NO}_3^-$  with  $\Delta^{17}\text{O} = 0$  [178]. Combining this information with a mass balance approach makes it possible to calculate the fractions of  $\text{NO}_3^-$  coming from the two different sources, i.e., atmospheric vs. terrestrial sources (particularly nitrification of either  $\text{NH}_4^+$  from wet deposition, soil  $\text{NH}_4^+$  pool, and/or fertilizer applications [182, 183]).

While the triple isotope approach ( $\delta^{15}\text{N}$ ,  $\delta^{17}\text{O}$  and  $\delta^{18}\text{O}$  in  $\text{NO}_3^-$ ) has been mostly applied to assess the contribution of atmospheric vs. biological sources for  $\text{NO}_3^-$  in streamwater to elucidate nitrogen loss pathways associated with leaching [176, 183], very few studies have applied it to investigate the occurrence of canopy nitrogen transformations. Guerrieri et al. [165••] measured multiple isotopes in bulk precipitation and throughfall water (i.e.,  $\delta^{15}\text{N}$  in  $\text{NH}_4^+$  and  $\delta^{15}\text{N}$ ,  $\delta^{18}\text{O}$  and  $\Delta^{17}\text{O}$  in  $\text{NO}_3^-$ ) collected at two Scots pine and two beech forests at contrasting levels of nitrogen deposition. Using this approach, the authors found that forests under high nitrogen deposition have a significant fraction of the nitrate in throughfall derived from nitrification in tree canopies (higher for beech (59%) compared to Scots pine (17%)), and less from atmospheric nitrogen deposition. Nitrification in tree canopies was also detected in a Mediterranean holm-oak forest, though these exposed to lower nitrogen deposition compared to the sites in the UK [166••]. If stable isotopes (particularly  $\Delta^{17}\text{O}$  in  $\text{NO}_3^-$ ) provide indications on biological transformations occurring in tree canopies, can we identify microbes responsible for them?

### Phyllosphere Epiphytes and Their Role in Processing Atmospheric Nitrogen Within Canopies

Forest canopies represent an important habitat (i.e., phyllosphere) for epiphytes and endophytes, which include lichens ([184–186] for a review) and microbes, i.e., archaea, bacteria, and fungi [187–190] for a review – Fig. 4).

The total extent of global phyllosphere habitat is thought to represent  $6.4 \times 10^8 \text{ km}^2$ , which could harbor  $10^{26}$  bacterial cells [190–192]. A strong association



**Fig. 4.** Example of life diversity in tree canopies. View of the diversity of epiphytes within the canopy of *Ficus tuerckheimii* as reported in Gotsh et al. 2015 (panel A). Phyllosphere microbes in the lower surface of a *Quercus robur* and a *Fagus sylvatica* leaves (panel B and C,

respectively). Image in the panel B was taken from Vacher et al. (2016). Image in the panel C was obtained by epifluorescence microscopy on DAPI-stained cells, showing bacteria (green dots) on leaf surface, along the main veins (copyright Rossella Guerrieri)

between leaf traits (such leaf mass per area, leaf thickness, leaf area and leaf nutrient, and pigments associated to photosynthesis) and bacterial [189••] and fungi [193••] phyllosphere communities was reported. Despite epiphytic microbes representing an important component of tree canopies [194, 195••], attention has been mostly directed to their role as pathogens, while we still do not know whether and how they affect nutrient cycling [187, 190]. Moreover, earlier studies were mostly based on the use of laboratory cultures, making difficult to actually characterize which microbial communities live in forest canopies. The advent of high throughput sequencing and recent *omic* techniques offer a great opportunity to take a snapshot of the microbes living in the phyllosphere to characterize community composition and to explore the functional role of taxa within these communities.

Several studies have provided information on species composition of microbial communities in the phyllosphere (cf., [188, 189••, 196]). Leaves often share a similar bacterial composition regardless of the position within the canopy [166••, 196], while leaf age and phenology (i.e., leaf development) and climate affect composition and diversity of the bacterial communities they host, though most results refer to the canopy of herbaceous species. More diverse bacterial communities in younger compared with older leaves were observed ([197, 198] on crop plants; [199••] in urban forests), likely associated with the decrease in nutrient availability from the host plant. Experimental increase in temperature and atmospheric CO<sub>2</sub> enhanced richness and diversity of bacteria in phyllosphere of *Galium alba* [200] and ‘Koshihikari’ rice plants [201]. Similar results were also observed in the case of phyllosphere of *Quercus ilex* trees subjected to drought [202].

Compared to microbial communities living in the soil, those in the phyllosphere experience more rapid (from diurnal to seasonal) changes in environmental conditions, including ultraviolet radiation, relative humidity, temperature, carbon, and nutrients availability [195••, 203•]. While effects of biotic and abiotic stress on the microbial communities have been discussed [187, 194], we are not aware of any investigations conducted to determine whether and how changes in nitrogen deposition affect abundance of microbial taxa associated with nitrogen transformations.

A proteomic approach allows researchers to detect enzymatic functions of bacteria in the phyllosphere, such as enzymes able to convert methanol to obtain carbon and assimilate NH<sub>4</sub><sup>+</sup> via glutamine synthetase [188, 204••]. Biological nitrogen fixation (the process that converts atmospheric N<sub>2</sub> to reactive nitrogen in the form of NH<sub>4</sub><sup>+</sup>) is mostly attributed to symbiotic nitrogen fixers found in root nodules. Yet, there is mounting evidence of the occurrence of nitrogen fixation in tree canopies of different forest ecosystems, mediated by free-living archaea and bacteria nitrogen fixers [205, 206] for a review] that are associated with foliage or epiphytes (e.g., [207–210••] in tropical forests, [211] in a Mediterranean forest, [212••, 213] in a temperate forests). Indeed, nitrogen fixation by microbes associated with epiphytes has been shown to be particularly relevant for old growth forests, characterized by the significant abundance of epiphytes in their canopy, such as in coastal temperate rain forests in British Columbia [214]. A recent study in the Niwot Ridge in the US [212••] showed N<sub>2</sub>-fixing acetic acid bacteria living as endophytes on limber pine needles. Moreover – and most importantly – the authors demonstrated that the fixed nitrogen diffused inside the needles and entered plant metabolism, as indicated by the increase in nitrogenase

activity. Recent studies showed that almost 50% of the nitrogen derived from nitrogen fixation was carried out above-ground on tree trunks and within tree canopies of tropical forests in French Guiana [209••] and in Brazil [210••]. Interestingly, canopy nitrogen fixation seems to increase with foliar phosphorous [208••, 210••] and is not limited by drought conditions [210••, 211].

Nitrogen fixation, however, may not be the only biological transformation occurring in tree canopies. A number of studies have provided evidence of nitrifying microbes harbored in the phyllosphere. Indeed, nitrifying bacteria were found within the apoplast of needles in a spruce forest in Germany subjected to high nitrogen deposition [215, 216], or archaeal *amoA* genes related to nitrification were found on foliar in a *Cryptomeria japonica* forest in Japan [174••]. Both archaeal and bacterial *amoA* genes were found on the phyllosphere of *Quercus ilex* in a Mediterranean forest, whose activity in processing atmospheric nitrogen deposition through nitrification within tree canopies was assessed by using  $\Delta^{17}\text{O}$  in bulk deposition and throughfall, as described in the previous chapter [166••]. The presence of  $\text{NO}_2\text{-N}$  and organic N-using fungi were observed on needles collected at top and intermediate branches of black spruce trees in Canada [116], which contributed also to significantly changing nitrogen fluxes at various canopy heights.

This information provides important evidence, yet site-specific, for the presence and activity of nitrogen-related microbes in the phyllosphere, but additional studies—either along climate and nitrogen deposition gradients or for different forest ecosystem types—are needed to better quantify those processes at larger scales. Moreover, extending the characterization of microbial communities to bulk deposition, throughfall and stemflow water other than the phyllosphere, would provide a deeper understanding of ecosystem nitrogen dynamics associated with microbial dispersion (e.g., in relation to the origin of air masses, see [217•]), interception by tree canopies and their release during a rainy event [218••], thus, contributing to enriching the microbial communities in the soil [133••]. Intrusion of dust from the Sahara Desert (i.e. air masses from northern Africa) has been shown to enrich the air mass with nutrients and allochthonous microbial components [208••], and changes both composition and diversity of bacterial communities [166••, 217•, 219]. Bittar et al. [133••] estimated a flux of  $1.5 \times 10^{16}$  microbial cells  $\text{ha}^{-1} \text{year}^{-1}$  to the soil from throughfall in a subtropical oak-cedar forest in Southeastern USA, which corresponded to a contribution of organic carbon from the phyllosphere to the soil of 0.6–2.3  $\text{kg ha}^{-1} \text{year}^{-1}$ . In a study carried out on a holm oak forest in Spain precipitation water and the phyllosphere shared some of the same bacterial taxa, which were not found in throughfall water, suggesting that tree canopies may act as a filter not only

for atmospheric chemical compounds, but also for microbes carried by precipitation [166••].

The canopy organic mat, which includes tree canopies and associated epiphytes, invertebrates, and microbes, has been acknowledged as ‘nutrient capital’ [220], particularly in tropical moist forest ecosystems. Indeed, dead canopy organic matter undergoes decomposition processes, which lead to the formation of the so-called ‘crown humus’ [221], which is commonly referred to as ‘arboreal soil’ [222] or ‘canopy soil’ [220]. Canopy soil has been less studied compared to soil in the forest floor, given the challenges associated with the sampling, though several studies have pointed to its ecological relevance [220, 222, 223••]. Indeed, canopy soil is a repository of nutrients (including nitrogen) epiphytes and other living organisms rely on, which either derives from decomposition and mineralization of the dead organic matter or retention of atmospheric deposition [222, 224, 225]. Mineralization and nitrification in canopy soils of a tropical montane forest in Ecuador were limited by nitrogen availability, suggesting that increases in nitrogen deposition can enhance nutrient cycling occurring in tree canopies [220]. Last, but not least, soils in tree canopies and in the forest floor are interconnected thanks to leaching of nutrients through throughfall and stemflow [223••, 226], but also via invertebrate species [227–229]. As suggested by Van Stan et al. [230••], throughfall can be seen like a ‘hydrological highway’ connecting the atmosphere to the soil, thus, allowing inorganic and organic nitrogen and biological materials (derived from plants, epiphytes and/or microbes) to be transferred to the soil.

## Conclusions

Back in 1971, Hill [231] stated “It has often been observed that we know much more about the sources, movement, and effects of air pollutants than we do about their fate”. Since then, significant progress have been made from different research communities to elucidate the fate of air pollutants (including reactive nitrogen) and the crucial role that tree canopies play in altering nitrogen inputs from the atmosphere by retaining, assimilating, and transforming nitrogen deposition before it reaches the soil. The complexity of living organisms and the role they play in nutrient cycling has been well studied in forest soils [232]. We have just started to unveil the high diversity in microbial life in tree canopies and we are still far from understanding their roles in nitrogen cycling. How to move forward? The following represents research areas we suggest should be prioritized for next steps in this field, encouraging multidisciplinary approaches across different research communities interested in – but not limited to – nitrogen cycling in forest ecosystems.

- We suggest that researchers develop improved approaches to obtain more robust estimates of total nitrogen deposition. Satellite observations and modeling approaches play a crucial role in increasing the spatial coverage of atmospheric reactive nitrogen concentrations and deposition data, but there are several critical points that need to be solved to improve estimates of both wet and dry deposition, which are both needed in order to estimate total deposition (ref. [65•, 233] for an overview). For instance, satellite-based estimates of  $\text{NH}_3$  deposition do not account for bi-directional fluxes during the interaction between the biosphere and atmosphere [234, 235]. Ground-based observations cannot be replaced entirely by remote sensing data since canopy nitrogen processes (and their contribution to the ecosystem nitrogen cycling) have to be quantified. Moreover, weighting of the land-use classes within grid cell used for processing satellite data may not be representative of the vegetation type in the site of interest, thus, leading to uncertainties in the deposition estimates [56, 233].
- It is important to increase the monitoring of atmospheric deposition in places that are under-represented in our understanding of atmospheric nitrogen deposition, such as in tropical regions or urban environments (the latter being hotspots of atmospheric deposition [36•, 74•]). We recommend greater use of ion exchange resin collectors [37, 38]. Establishing an intensive monitoring network in the tropics (and other under-represented region in the world) is paramount to achieving a better understanding of the ecological consequences of increasing nitrogen deposition on nitrogen cycling and trade-offs between canopy and soil processes, as well as forest carbon and water fluxes. Moreover, including stable isotope analyses, particularly the use of  $\Delta^{17}\text{O}$ , in addition to quantification of nitrogen fluxes would greatly further our understanding of the impacts of atmospheric  $\text{NO}_3^-$  deposition on forest ecosystems and elucidating processes occurring during atmospheric-forest canopy interactions.
- Though more challenging from logistic and economic standpoints, we need to maintain current and establish more canopy nitrogen manipulation experiments in different forest ecosystems, particularly those where rates of nitrogen deposition are predicted to increase over the next several decades. Next-generation manipulation experiments should also simulate the increase in dry nitrogen deposition, particularly ammonia [236] or a reduction of nitrate deposition (i.e., particularly wet deposition), and explore the interactions between changes in nitrogen deposition and extreme climate events. Moreover, in addition to the response of trees, new experiments should include measurements of microbes in both the phyllosphere and rhizosphere.
- We recommend greater quantification of the relevance of canopy nitrogen uptake and canopy production—via microbial nitrification and fixation or biological activity by insects (e.g., insect frass) and trees themselves (i.e., e.g., pollen production, plant debris)—to better understand whole ecosystem nitrogen cycling. Recent studies highlighted the substantial contribution of asymbiotic nitrogen fixation in the phyllosphere [210••]. However, it remains to be estimated how much of this process contributes to ecosystem-scale biological nitrogen fixation, particularly in relation to increases in nitrogen inputs from anthropogenic activities, which could affect also tropical regions—where most of the studies on nitrogen fixation have been conducted so far [237]. We are far from understanding dynamics of nitrification in tree canopies—its magnitude, how it is affected by climate and nitrogen deposition and in which proportion it contributes to increase  $\text{NO}_3^-$  concentrations in soil solution. Providing robust estimates of canopy nitrogen uptake (and assimilation) and accounting for biological transformations within tree canopies can greatly improve estimates of total nitrogen deposition (e.g., through canopy budget model) and also improve the contribution of nitrogen input (from biological fixation and anthropogenic nitrogen deposition) to forest carbon sequestration, as represented in process-based [238] and terrestrial-biosphere models [239].
- We need to enhance our understanding of tree canopy-soil interactions. Tree canopies and soil are more interconnected than we think, as what is happening in the canopy (in terms of atmospheric nitrogen retention and uptake by foliage, branches and life they harbor and biological transformations by microbes in the phyllosphere) has the potential to affect soil microbial communities (via throughfall water ‘highway’ [230••]), and hence biogeochemical processes throughout a forest ecosystem [240••]. The advent of next-generation sequencing technologies [241] has increased our ability to study microbial communities (metagenomic) and their function (metatranscriptomic and metaproteomic) directly in the environment they live. A great example is the study by Delgado-Baquerizo et al. [232] mapping soil bacterial communities for different vegetation systems worldwide using metagenomic analyses. This recent development of new techniques is an exciting opportunity for forest ecologists to go beyond the greening of the canopies and their role in regulating carbon and water exchanges with the atmosphere and to assess their contribution to nutrient cycling. This means taking a more holistic approach, which explores i) the important contribution of forest water as input not only for nutrients, but also microbes; ii) the metabolic and functional role of microbes in the phyllosphere and their associations with their host trees. Mapping phyllosphere and forest water microbial communities, moreover, could represent an important

addition to the very challenging earth microbiome project, the global collaborative effort to characterize microbial life on Earth (<https://www.earthmicrobiome.org/publications/>).

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**Declarations**

**Conflict of Interest** Rossella Guerrieri, Pamela Templer, and Federico Magnani declare that they have no conflict of interest.

**Human and Animal Rights** This article does not contain any studies with human or animal subjects performed by any of the authors.

**Appendix**

**Stable isotope terminology and measurements**

Natural abundance of stable isotope composition of plant or nitrogen compounds in water samples are expressed as a ratio

relative to an internationally accepted reference standard (Table 2), which is referred to as stable isotope composition (delta notation,  $\delta$ ):

$$\delta^{xx}E = 1000 \times \left( \frac{R_{sample}}{R_{standard}} - 1 \right) \tag{1}$$

where  $E$  is the element of interest and “xx” is the mass of the rarest (and heavier) isotope in the abundance ratio (e.g.,  $^{15}\text{N}$ ,  $^{18}\text{O}$  or  $^{17}\text{O}$  in this review) and  $R$  is the abundance ratio of the two isotopes of interest (e.g.,  $^{15}\text{N}/^{14}\text{N}$ ,  $^{18}\text{O}/^{16}\text{O}$  or  $^{17}\text{O}/^{16}\text{O}$  for this review). The isotope composition of a given sample is indicated as  $\delta^{15}\text{N}$ ,  $\delta^{18}\text{O}$  or  $\delta^{17}\text{O}$ , and it can be obtained through mass spectrometer analyses – albeit sample preparation, which can differ depending on the sample type and the isotopic composition of interest. Note that the absolute  $R$  of the sample is very small (a few part per thousand). For this reason the isotopic composition is expressed in ‘per mill’ notation (‰) – which, hence, is not a unit.

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**Table 2** The element, their isotopes and percent abundance, isotope abundance measured and international standard considered. We also mention the sample types where used in the examples provided in chapters 4 and 5 of the review and references the readers can refer to for more details regarding methodological aspects (sample preparation and measurements, which is particular relevant in the case of water samples).

Element	Isotope	Percent abundance	Isotope abundance	Standard	Sample	Reference
N	$^{14}\text{N}$	99.63	$^{15}\text{N}/^{14}\text{N}$	$\text{N}_{2\text{-atm}}$	Foliar and tree ring samples, atmospheric nitrogen deposition	[105, 160, 162••, 164]
	$^{15}\text{N}$	0.3663				
O	$^{16}\text{O}$	99.759	$^{18}\text{O}/^{16}\text{O}$ $^{17}\text{O}/^{16}\text{O}$	V-SMOW	Foliar, nitrogen compounds in bulk, throughfall deposition and streamwater	[160, 162••, 177, 178••]
	$^{17}\text{O}$	0.037				
	$^{18}\text{O}$	0.204				

Note.  $\text{N}_{2\text{-atm}}$  and V-SMOW indicates atmospheric  $\text{N}_2$  and Vienna-SMOW (the latter available from the IAEA). Table was modified from Dawson T.E., Mambelli S., Plamboeck A.H., Templer P.H., Tu K.P. Stable isotopes in plant ecology. *Annu. Rev. Ecol. Syst.* 2002; 33:507–59.

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