

Reply to “No pervasive relationship between phyllosphere nitrifiers abundance and canopy nitrification in European forests”

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Forest canopies have long been recognized for their significant role in nitrogen (N) cycling¹⁻⁶. Since the first evidence of nitrifiers colonizing the phyllosphere of Norway spruce back in 2002⁷, nitrification has been discussed as a key biological process in the interaction between atmospheric N and tree canopies⁸⁻¹². However, its relevance at the ecosystem scale has been rarely elucidated. Guerrieri et al. 2024¹³ (from now on, G2024) quantified the contribution of biological nitrification to ecosystem N fluxes and demonstrated the presence of nitrifiers in the phyllosphere of *Fagus sylvatica* and *Pinus sylvestris* forests across a broad environmental gradient in Europe. G2024 found that on average $32 \pm 25\%$ of the total NO_3^- in throughfall (TF) derived from canopy nitrification, with a range of the fraction of NO_3^- coming from biological transformation (f_{Bio}) from 4% to 80%. f_{Bio} was higher in *Fagus sylvatica* than *Pinus sylvestris*, and it increased with atmospheric N deposition. When accounting for f_{Bio} in TF fluxes, G2024 reported that gross canopy nitrification (GCN) added 0.40-4.97 kg N ha⁻¹ yr⁻¹ (*Fagus sylvatica*) and 0.21-3.23 kg N kg N ha⁻¹ (*Pinus sylvestris*) to the NO_3^- from atmospheric deposition.

Recently, Duan et al. (this Comment) challenged these findings, based on the re-analyses of data presented in G2024. They found overall a weak relationship between f_{Bio} or GCN (estimated from stable oxygen isotopes and N fluxes) and abundance of archaeal and bacterial nitrifiers (from quantitative PCR). The authors identified three limitations in G2024 that could contribute to explaining the lack of the relationship. While the points raised by Duan et al. are relevant, we show that they do not provide compelling evidence disproving the occurrence of GCN as quantified in G2024.

An important premise is needed. G2024 quantified the process and its contribution in terms of N flux integrated over the growing season. However, the molecular analyses did not allow to ascertain when nitrifiers were active in carrying out the process. For instance, nitrifiers may be more active in processing atmospheric N during a dry period, when dry deposition can be retained and remains on leaf surfaces for longer¹². The mass balance approach based on $\Delta^{17}\text{O}$ in G2024 detects the biologically derived NO_3^- cumulated over days or months, that is washed off the canopies during a precipitation event, thus adding on the atmospheric input of NO_3^- in TF. This can lead to a decoupling between when the process occurs and when it is detected. Hence, it is not surprising that canopy nitrification and nitrifiers abundance show a weak correlation.

Limitation 1: Stemflow neglected in forest ecosystem fluxes. Duan et al. argued that one of the possible reasons for weak relationship between GCN and abundance of nitrifiers can be

the exclusion of stemflow (SF) flux in G2024. The flux reaching the soil via SF would greatly depend on bark features as well as canopy interception, besides precipitation. For instance, SF in *Fagus sylvatica* can be higher than in *Pinus sylvestris*, due to its smoother bark and funnel-shaped crown¹⁴. We focused on TF as it is generally a higher flux than SF, particularly during summer^{3,15} and it is the flux most frequently considered in studies assessing the role of tree canopies in retaining or releasing N⁶. To the best of our knowledge, only one study reported presence of nitrifiers on stem bark¹⁶, but the process itself was not quantified. Even if nitrifiers were present on the bark and nitrification occurred, we would have underestimated GCN, but this does not disprove the occurrence of the process. For example, at sites G2024 observed the highest f_{Bio} , i.e., *Fagus sylvatica* in Hoeilaart and *Pinus sylvestris* in Brasschaat, the long-term (1996-2023) contribution of SF to total precipitation (TF+SF) was 1% and <6.4%, respectively (Verstraeten, personal communication), which is in line with data measured across the ICP Forests Level II sites (17; Waldner et al. personal communication). For *Fagus sylvatica* in Hoeilaart we could calculate the f_{Bio} and GCN for 2016. NO₃ flux in stemflow was 0.2 kg ha⁻¹ yr⁻¹ (TF was 7.2 kg ha⁻¹ yr⁻¹), f_{Bio} was 0.17, leading to a GCN of 0.034 kg ha⁻¹ yr⁻¹. Bacterial nitrifiers in stemflow water (both bacterial *amoA* and bacterial *nirB*) were present (10^{7.3} and 10^{5.1} copy number per ng of DNA extracted, respectively), while archaeal nitrifiers (*amoA*) were not detected. Again, this may underestimate GCN, but it does not disprove the key message in G2024 that nitrification occurs in the phyllosphere.

Limitation 2: Neglecting nitrification by fungi and comammox and other components in the phyllosphere. Duan et al. suggested that the lack of relationship between GCN and nitrifiers could be due to the exclusion of fungal and comammox nitrifiers as well as of flowers and fruits/cones as phyllosphere components. Assuming fungal and comammox nitrifiers were present and actively involved in nitrification, our study would underestimate the absolute abundance of nitrifiers in the phyllosphere, but again, this argument does not go against the occurrence of the process.

Flowers and fruits/cones are important components of the phyllosphere, but their phenology is shorter compared to the foliage. Flowering and fructification normally occur at the beginning of the growing season^{18,19}; particularly in the case of *Fagus sylvatica*, fructification alternates mast years with low fruit production years²⁰. Whether nitrifiers can be present and be active in pine cones and seed capsules remains to be explored. Our experimental design aimed at capturing the biological process at the maximum of canopy cover (in terms of leaf

area index) and of host tree physiological activities. Assessing the biological transformation of atmospheric N following the phenology of tree canopies and their components should be the next important step, but it was not the goal of the study in G2024, which focused on spatial rather than seasonal canopy nitrification.

Limitation 3: Uncertainties related to the isotope approach. The limitation raised regarding the isotope approach neglects key theoretical understanding on isotope fractionation processes involving NO_3^- . Even though the 0.52 coefficient may vary in the relationship between $\delta^{17}\text{O}$ and $\delta^{18}\text{O}$ (the range reported in the literature is 0.5-0.53²¹), it remains that biological transformations are associated with mass dependent isotope fractionations, which lead to $\Delta^{17}\text{O}$ equal to zero²². Whereas atmospheric NO_3^- is mostly related to mass independent isotope fractionations during photochemical reactions leading to an excess of ^{17}O , with $\Delta^{17}\text{O} > 0$ ²². Guerrieri et al. 2015¹¹ compared the estimate of f_{Bio} using both $\Delta^{17}\text{O}$ and $\delta^{18}\text{O}$. While the magnitudes differed, both methods indicated the occurrence of canopy nitrification. The discussion should rather focus on capturing seasonal changes in atmospheric NO_3^- - $\Delta^{17}\text{O}$ and in NO_3^- collected directly on leaf surfaces, which can both strengthen the estimate of f_{Bio} and be more directly linked to the abundance of nitrifiers.

We are grateful to Duan et al. for the positive comments on G2024 and for highlighting relevant areas of improvements. We welcome additional evidence that supports or challenges the occurrence of canopy nitrification and its relevance in the ecosystem N flux. We hope this discussion will inspire further research into the microbes inhabiting forest canopies and their role in the atmosphere-tree canopy interactions, employing a multidisciplinary and multi-scale approaches. There is no doubt that in-situ (leaf surface) nitrification should be quantified, perhaps under manipulative conditions (e.g., NH_3 enhancement systems²³ and/or using labeled N and O isotopes) coupled with omics approaches^{24,25} based on RNA analyses, to directly link the process to active nitrifiers and their conditions. We conclude that the overall weak relationships found between f_{Bio} or GCN and abundance of nitrifiers should not be over-interpreted, since none of three limitations highlighted by Duan et al. is likely to challenge our main conclusion.

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206 **Data availability**

207 No data are included in this paper.

208 **Code availability**

209 No data analysis is included in this paper.

210

211 **Author contributions**

212 RG wrote the first draft with feedback from MM, JC and SM. All the co-authors provided

213 feedback on the first draft and agreed on the final content of the reply.

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