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Occurrence of nitrification in tree canopies of European forests as proved by oxygen isotope and metabarcoding analyses

Fluxes and chemical composition of precipitation is substantially changed after passing through tree canopies, particularly in the case of atmospheric reactive nitrogen compounds. Nitrogen retention and uptake or leaching of dry nitrogen compounds during precipitation events is the main mechanisms proposed to explain differences in nitrogen fluxes between bulk deposition (BD) and throughfall (TF). Yet, many studies since the early 1950s have demonstrated that forest canopies represent an important habitat (*i.e.*, phyllosphere) for microbial communities (including archaea and bacteria), but whether they play a role in nutrient cycling, particularly nitrogen, is not well studied. In a previous study we provided isotopic evidence that between 17% (Scots pine) and 59% (beech) of nitrate in TF derived from canopy nitrification in two UK forests at high nitrogen deposition. This finding strongly suggested that forest canopies are not just passive filters for precipitation water and dissolved nutrients, and that the microbial life hidden within them can be responsible for transforming atmospheric nitrogen before it reaches the soil. We extended the isotopic approach at the European scale, and combined it to next-generation sequence analyses with the aim of elucidating canopy nitrification and identify phyllosphere microbes responsible for it. In this study we: (i) estimated the relative contribution of nitrate derived from biological canopy nitrification (fBio) *vs.* atmospheric deposition by using stable oxygen isotope composition ($\delta^{18}O$ and $\delta^{17}O$) of nitrate in rainfall and throughfall water; (ii) quantified the functional genes related to nitrification, and finally (iii) characterized the microbial communities harboured in the phyllosphere for two dominant tree species in Europe (*Fagus sylvatica* L. and *Pinus sylvestris* L.) using metabarcoding techniques. We considered ten sites included in the European ICP Forests monitoring network, chosen along climate and nitrogen deposition gradients, spanning from Fennoscandia to the Mediterranean. We found that on average 23% of the nitrate reaching the soil via throughfall derived from canopy nitrification, with fBio ranging from 0 at the low nitrogen deposition site (in Sweden) up to 75% at the high nitrogen deposition forest sites (in Belgium). The occurrence of the biological transformation was confirmed by the presence of nitrifying microbes on leaf surfaces, both archaea and bacteria, as assessed through quantitative PCR and 16S sequence analyses. Quantification of canopy nitrification in this study as well as of canopy of N2 fixation and N2O emissions from leaves in previous studies call for a reconsideration on key processes underpinning nitrogen cycling, which should no longer exclude forest canopies and microbial life hidden within them.

Parole chiave: phyllosphere, nitrogen cycling, stable isotopes, next-generation sequence analysis, nitrogen deposition, Scots pine, beech

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