

Substantial contribution of tree canopy nitrifiers to nitrogen fluxes in European forests

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Supplementary information for:

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Supplementary Methods

Text S1. Limitations of the proposed approach for estimating Gross Canopy Nitrification (GCN)

We are aware that the approach considered might have some limitations, as it is based on isotopic measurements in NO_3^- in forest water samples rather than directly on leaf surfaces. Here we discuss those limitations and address them, based on evidence in the literature and previous experiments that we carried out at four ICP Forests sites.

a) Increase in NO_3^- in BD and TF water (in the field or after collection) due to biological transformation.

Earlier studies (e.g., [1] and references therein) reported that concentrations of N compounds vary in both TF and BD water in the field. Previous studies (e.g., [2]) assumed that nitrification would not occur in the open-field water collectors (BD), because these samples are not expected to be colonised by nitrifiers. Although nitrifiers can be present in rainwater and throughfall water ([3] and Supplementary Figure S1 in this study), they represent a very small part of the whole microbial community (<0.1 % in most of the sites included in the study, data not shown). If microbial transformations had already occurred in the water collectors at our sites, however, their contribution would have been small compared to the process occurring in tree canopies for three reasons, which we discuss below.

1) We searched for studies assessing the differences between nitrogen fluxes estimates based on two approaches: water collection with the conventional approach (as in this study) and the exchange resins (IER) method e.g., [2, 4]). In this latter case, NO_3^- and NO_4^+ are directly trapped in the anion/cation resins, thus minimising the risk of microbial colonisation, and hence biological transformations. Overall the two approaches led to similar estimates of N deposition [4] or slightly higher NO_3^- -N values

were reported in the case of the conventional approach vs. IER, with magnitude of $+0.04 \pm 0.12$ [2] and $+0.27 \pm 0.19 \text{ kg ha}^{-1} \text{ yr}^{-1}$ [5]. The difference values above were subtracted to the measured TF NO_3^- -N deposition measured at our sites and the obtained values were multiplied for the f_{Bio} , so to obtain corrected GCN values, after accounting for potential nitrification in the water samples. By using the lower and higher correction factors, we obtained corrected mean GCN values of 1.74 ± 1.75 and 1.76 ± 1.76 , respectively, whereas actual mean atmospheric fluxes ranged from 3.19 ± 1.11 and 3.22 ± 1.12 .

2) We present the results from tests conducted in 2010 to examine directly the issue of biological transformations in the water samples while in the field or after collection. Specifically, at four ICP Forests sites in Germany (Supplementary Tables S1-S2) TF water was collected the day after a rain event. Filtered and unfiltered samples were kept in PVC bottles and subjected to temperatures of 4, 8, 20, and 30 °C in the laboratory to simulate different field conditions. Changes in the pH and in the NH_4^+ , NO_3^- , and TN concentrations in TF water were monitored for up to 85 days after collection. The results indicated that N concentrations changed after 2 weeks, particularly for the unfiltered water. The changes were generally larger at higher storage temperatures, and the concentrations changed more strongly over time for NH_4^+ and TN than NO_3^- , particularly for the unfiltered samples (Supplementary Figure S3). Conversely, NO_3^- concentrations in the filtered samples generally remained highly stable for several weeks. pH was also quite stable during the experiment at most of the sites (Supplementary Figure S4) and particularly for the temperature water samples were stored in this study (2-4 °C). If nitrification systematically occurred in the water in the field, one would expect a reduction in NH_4^+ concentration, an increase in NO_3^- concentration, and large changes in pH, which was not the case in the test

(Supplementary Figures S3 and S4, Supplementary Table S2). Higher variability in different N form concentrations and pH was observed at only 1 site (Lüss), but for the 20° and 30°C temperature levels and after 2 weeks. Samples were collected every two weeks at the majority of our sites, particularly where f_{Bio} and GCN were higher, thus minimising the influence of biological transformations in the water. Finally, we did not observe large changes in N concentrations and pH in the filtered and unfiltered samples maintained at 2-4 °C (Figures S3 and S4), i.e. the temperature at which samples were stored, after collection and/or filtering at the sites where GCN was estimated. In addition, the pH of the water measured at the beginning of the experiment showed values between 4.2 and 5.6 depending on the site, which are acidic conditions where autotrophic nitrification does not typically occur [6]. Measured pH values during the experiment remained below the optimum pH for most nitrifiers, i.e., 7.5 for ammonia oxidizers (Nitrosomonas, Nitrosospira and Nitrososphaera) and 7.0 for nitrite oxidizers (Nitrospira and Nitrobacter) [7,8]. Nitrification under low pH can also be carried out by acid-tolerant nitrifiers, such as species of archaea “*Candidatus (Ca.) Nitrosotalea*” [9] or bacteria “*Candidatus (Ca.) Nitrosoglobus terrae*” [10], “*Candidatus (Ca.) Nitrosacidococcus tergens*” [11] and Nitrosococcus-like species [12]. None of these acid-tolerant nitrifiers has been detected in the water samples of the studied sites (bulk deposition and TF) although we cannot rule out that the unclassified AOA observed in the phyllosphere (Figure 4 in the main text) and in the water samples (data not shown) could also potentially be active at lower pH. However, the very low abundance of nitrifiers (<0.1% of the whole communities in BD and TF water at most sites, data not shown) and the mostly unfavorable conditions for them support the probable absence of nitrification in the collectors.

3) The calculation of f_{Bio} included both BD and TF, so any transformation occurring in the water (in the field or even after collection) will already have been accounted for. We cannot exclude microbial transformations after collection, particularly for the sites where filtering was conducted a few months later, e.g. sites in France and Italy (Supplementary Table S3). If transformations did occur, however, large changes in the isotopic signatures and high f_{Bio} would have been observed, which was not the case. For the other sites, filtering was conducted between 1 and 4 weeks after sampling. A limitation of the isotopic approach, though, could be that any signals of microbial activity during dry days or at sites with low N deposition (where canopy retention was the dominant ecological process) could have been diluted by the discharge of atmospheric N deposition during a rain. The method by which the TF water was processed, e.g. by pooling water from multiple rains within a month, may also add uncertainty, which could partially account for why occasional values <0 or >1 were obtained for f_{Bio} from biological activity and the atmosphere, respectively (Extended Data Table 4).

b) Influence of soil NO and N₂O emissions and interaction with forest canopy.

A number of studies have shown that soil NO and N₂O emissions can be particularly relevant in conifer forests and under high N deposition. Soil NO_x was proposed to contribute to NO₃⁻ collected in precipitation water, as indicated by $\Delta^{17}\text{O}$ values in NO₃⁻ as low as +11‰ [13]. We exclude that NO₃⁻ washed out from tree canopies could partially originate from soil NO for two main reasons: firstly, soil emitted NO_x have been reported to have $\delta^{15}\text{N}$ values ranging between -59.8‰ to -19.9‰, ([13] and reference therein). As reported in the Extended Data Table 3 and Extended Data Figure 2, $\delta^{15}\text{N}$ values in NO₃⁻ for both BD and TF ranged in most of the sampling date between -10 and +20‰, with only a few values lower (more negative) than -10‰. On average,

the difference between TF and BD for $\delta^{15}\text{N}$ in NO_3^- was $+2.5\text{‰} \pm 12.3\text{‰}$, with only 7 out of 21 point measurements where $\delta^{15}\text{N}$ in TF had negative values vs. positive $\delta^{15}\text{N}$ in BD. This suggests a small contribution of soil NO_x to NO_3^- collected particularly in TF water. Last, but not least, O_3 is expected to be lower under tree canopies (as shown in [14]), hence an accumulation of biogenic NO and NO_2 and a slow conversion to NO_3^- could be expected underneath tree canopies. Secondly, the contribution of soil NO_x emissions compared to atmospheric deposition could be small particularly at the high nitrogen deposition sites. Pilegaard et al. (2006) [15] showed that soil NO and N_2O emissions were higher in conifer than deciduous forests and relationship between soil emissions and N deposition was significant only in the case of conifer forests. However, the contribution of soil $\text{N}_2\text{O}+\text{NO}$ emissions was of a lower order of magnitude of N deposition (i.e., at the highest N deposition site, atmospheric input was 5 times higher than that coming from soil $\text{NO}+\text{N}_2\text{O}$ emissions (ref. Figure 2 in [15])).

Text S2. Molecular analyses

Preparation of DNA library for sequence analysis with Illumina platform

We targeted the V5-V6 region of the microbial 16S rRNA gene using a cyanobacteria-excluding primer (16S primers 799F-1115R) to exclude chloroplastic DNA [16]. We used a forward primer with an overhang adapter, TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**AACMGGATTAGATACCKG**, and a reverse primer with an overhang adapter, GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**AGGGTTGCGCTCGTTG**, as recommended in the Illumina manual for 16S Metagenomic Sequencing Library Preparation, where the bold text indicates locus-specific sequences. The first PCR

reaction had a total volume of 25 μ L consisting of: 1-2 μ L of Amplicon PCR Forward Primer (5 μ M), 1-2 μ L of Amplicon PCR Reverse Primer (5 μ M), 2.5 μ L of microbial DNA, 12.5 μ L of 2 $\times$ KAPA HiFi HotStart ReadyMix (Kapa Biosystems), and MilliQ water. Reactions were conducted following the Illumina procedure: 95 $^{\circ}$ C for 3 min and then 34 cycles of 95 $^{\circ}$ C for 30 s, 55 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 30 s, with a final extension at 72 $^{\circ}$ C for 30 min. LabChip electrophoresis indicated that the amplicons were 400 base pairs (bp) long. The reactions were purified using CleanPCR beads (CleanNa) and ethanol to remove free primers and/or primer dimers. A second PCR was performed with the cleaned PCR product as template to attach dual indices and Illumina sequencing adapters using the Nextera XT Index primers. Individual 50- μ L reactions were performed for each sample, with reagents and conditions as described above, except the reactions were cycled for only eight cycles. Multiplexed 16S libraries were prepared by mixing 5 μ L of each reaction at a concentration of 8 pM with 30% PhiX (the internal DNA control from Illumina). The DNA was sequenced using an Illumina MiSeq platform (San Diego, CA, USA) with a 500-cycle cartridge. DNA extraction, amplicon preparation, and sequence analysis were conducted at Servei de Genòmica i Bioinformàtica (Universitat Autònoma de Barcelona, Spain).

Analyses of 16S rRNA gene amplicons

Illumina paired-end raw sequences were processed using the UPARSE pipeline [17]. Forward and reverse sequence pairs were merged, and low-quality paired sequences were discarded by setting a minimum length of 330 bp and an expected error >0.5 [18]. Sequencing errors and chimeras were removed using the UNOISE algorithm [19] that clustered sequences into operational taxonomic units (OTUs) at 100% identity, i.e. zero-radius OTUs (ZOTUs). Taxonomic assignment used the naive Bayes scikit-learn

classifier implemented in QIIME2 [20] and the SILVA 132 database [21]. Chloroplastic, mitochondrial, and unclassified sequences (<2% of all sequences) were excluded from further analyses. We obtained totals of 1 960 737 reads and 21 394 ZOTUs, with 651 microbial genera identified across all foliar samples. Both the sequence identity of the ZOTUs that matched known autotrophic ammonia and nitrite oxidisers [22, 23] and PICRUST2 [24] for predicting metagenome functions were conducted for detecting microorganisms potentially involved in nitrification in the phyllosphere. A similarity of at least 97% was used for the ZOTUs that required species-level identification (*Nitrospira inopinata* and *Burkholderia insulsa*).

qPCR

We used real-time quantitative polymerase chain reactions, qPCRs, to quantify targeted genes on a LightCycler 480 (Roche, Mannheim, Germany). Bacterial *nifH* genes were amplified using the primers *nifHF/nifHR* [25], bacterial *amoA* genes were amplified using the primers *amoA2F/amoA1R* [26], archaeal *amoA* genes were amplified using the primers *amoA19F/amo643R* [27, 28], and *nxrB* genes were amplified using the primers *nxrB1F/nxrB1R* [29], and *nirK* genes were amplified using the primers *nirK876/nirK1040* [30]. Triplicate dilution series (10^2 - 10^8) of purified PCR amplicons from the genomic DNA (gDNA) of *Nitrospira* sp., from the environmental clone N3-16 [AB622272] [31], and from genomic DNA of *Nitrobacter winogradskyi*, and from gDNA of *Sinorhizobium meliloti* served as quantification standards for bacterial *nifH*, *amoA*, archaeal *amoA*, *nxrB*, and *nirK*, respectively. Individual PCR reactions had a total volume of 10 μ L consisting of: 5 μ L of SYBR Green qPCR Master Mix (Bimake, Huston, USA), 0.25 μ L of each primer (10 μ M) (Metabion, GmbH, Germany), 0.25 μ L of dimethyl sulfoxide (Sigma-Aldrich, St. Louis, USA), 3.5 μ L of DH_2O (PCR grade, Roche), and 2.5 μ L of template DNA (4 ng μL^{-1}). Cycling conditions were 95 °C for 5

min followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s for the bacterial *amoA* gene. Conditions were the same for all the genes except for annealing temperature that was 55 °C for archaeal *amoA* and *nifH* and 58.5 °C for *nirK* and *nxB* genes. Melting-curve analysis of the PCR products was conducted following each PCR run to confirm that the fluorescent signal originated from the PCR products and not from dimers or other artefacts. Samples and standards were amplified in triplicate, and several negative controls were included in each qPCR run. R^2 for the calibration curve was 0.99 for the three genes, and the slopes were -3.44 for *nifH* and bacterial *amoA*, -3.51 for archaeal *amoA*, -3.32 for *nxB* and -3.41 for *nirK*. Amplification efficiencies, calculated as $E = [10^{(-1/\text{slope})} - 1] \times 100$, were 95% for *nifH* bacterial *amoA*, 93% for archaeal *amoA*, 100% for *nxB* and 96% and *nirK*. Gene copy numbers were normalised to nanogrammes of extracted DNA. Sequences of oligonucleotides for all functional genes are reported in the Table S4.

Table S1. Variables of climate and nitrogen deposition measured at the sites where the experiment assessing changes in the concentrations of nitrogenous compounds in the throughfall water over time was conducted in 2010. See Text S1 for more details on the experiment, and see Figures S3 and S4 for the results. Mean annual temperature (T_a) and precipitation (P_a) refer to the years 1991-2020, while annual atmospheric deposition of NH_4^+ -N, NO_3^- -N and total nitrogen (TN) refer to 2020.

Site	Tree species	T_a (°C)	P_a (mm)	NH_4^+		NO_3^-		TN	
				BD	TF	BD	TF	BD	TF
Gottinger Wald	<i>Fagus sylvatica</i>	8.1	806	2.84	3.95	2.31	3.62	5.77	9.59
Harste	<i>Fagus sylvatica</i>	9.1	743	6.34	5.11	3.59	4.75	8.2	15.3
Solling	<i>Picea abies</i>				8.83		6.69		19.0
Lüss	<i>Fagus sylvatica</i>	9.3	815	4.02	4.16	2.64	2.72	7.48	9.06

Table S2. Results from the experiment assessing changes in the concentrations of nitrogenous compounds in the throughfall water over time conducted in 2010 at 4 ICP Forest sites in Germany, as described in the Text S1. After collection, the TF water was subjected to different temperatures (4, 8, 20, and 30 °C) for up to 85 days from sample collection, to simulate conditions in the field (4, 8, 20 and 30 °C) and when samples are stored in the laboratory (in the dark at 2-4 °C). We report the mean $\pm$ standard deviation calculated over n=2 replicates (with some exceptions). Results are also shown in Figures S3-S4.

Site	Species	Day	T	pH		NH ₄ ⁺ -N (mg N L ⁻¹)		NO ₃ ⁻ -N (mg N L ⁻¹)		TN (mg N L ⁻¹)	
			(°C)	Filtered	Unfiltered	Filtered	Unfiltered	Filtered	Unfiltered	Filtered	Unfiltered
Göttinger Wald	Fagus sylvatica	0	4		5,38	n/a	0,70 $\pm$ 0,00	n/a	1,28 $\pm$ 0,00	n/a	2,20 $\pm$ 0,00
		2			4,99		0,72 $\pm$ 0,01		1,30 $\pm$ 0,01		2,22 $\pm$ 0,01
		5			5,49		0,74 $\pm$ 0,01		1,26 $\pm$ 0,00		2,20 $\pm$ 0,00
		13			5,85		0,76 $\pm$ 0,04		1,25 $\pm$ 0,00		2,27 $\pm$ 0,06
		27			5,9		0,81 $\pm$ 0,10		1,29 $\pm$ 0,03		2,26 $\pm$ 0,02
		85			5,92		0,99 $\pm$ 0,21		1,34 $\pm$ 0,01		2,57 $\pm$ 0,12
		0	20	5,23	5,38	0,69 $\pm$ 0,00	0,70 $\pm$ 0,00	1,25 $\pm$ 0,00	1,28 $\pm$ 0,00	2,15	2,20 $\pm$ 0,00
		2		5,04	5,53	0,67 $\pm$ 0,01	0,71 $\pm$ 0,01	1,28 $\pm$ 0,00	1,31 $\pm$ 0,01	2,15 $\pm$ 0,01	2,30 $\pm$ 0,11
		5		5,05	5,76	0,66 $\pm$ 0,00	0,72 $\pm$ 0,01	1,28 $\pm$ 0,01	1,28 $\pm$ 0,02	2,18 $\pm$ 0,02	2,20 $\pm$ 0,03
		13		5,13	5,85	0,60 $\pm$ 0,03	0,85 $\pm$ 0,16	1,27 $\pm$ 0,01	1,25 $\pm$ 0,02	2,19 $\pm$ 0,04	2,41 $\pm$ 0,26
		27		4,9	5,73	0,55 $\pm$ 0,01	0,98 $\pm$ 0,54	1,27 $\pm$ 0,01	1,26 $\pm$ 0,02	1,99 $\pm$ 0,02	2,41 $\pm$ 0,47
		85		4,74		0,40 $\pm$ 0,02		1,28 $\pm$ 0,00	1,58	1,78 $\pm$ 0,02	2,67
Harste	Fagus sylvatica	0	4		5,82	n/a	0,79 $\pm$ 0,00	n/a	0,98 $\pm$ 0,00	n/a	2,16 $\pm$ 0,00
		2					0,83 $\pm$ 0,00		0,99 $\pm$ 0,00		2,20 $\pm$ 0,00
		5			5,88		0,85 $\pm$ 0,00		0,98 $\pm$ 0,01		2,18 $\pm$ 0,03
		13			6,19		0,88 $\pm$ 0,03		0,96 $\pm$ 0,00		2,23 $\pm$ 0,04
		27					0,93		1,00		2,23
		0	20	5,1	5,82	0,79 $\pm$ 0,00	0,79 $\pm$ 0,00	0,96 $\pm$ 0,00	0,98 $\pm$ 0,00	2,15 $\pm$ 0,00	2,16 $\pm$ 0,00
		2				0,73 $\pm$ 0,01	0,84 $\pm$ 0,02	0,96 $\pm$ 0,00	0,99 $\pm$ 0,01	2,09 $\pm$ 0,01	2,22 $\pm$ 0,06
		5		5,24	5,85	0,67 $\pm$ 0,04	0,84 $\pm$ 0,07	0,98 $\pm$ 0,01	0,98 $\pm$ 0,01	2,06 $\pm$ 0,00	2,23 $\pm$ 0,09
		13		5,12	6,21	0,47 $\pm$ 0,08	0,61 $\pm$ 0,15	0,95 $\pm$ 0,01	0,96 $\pm$ 0,01	1,94 $\pm$ 0,13	1,90 $\pm$ 0,16
		27		4,96	6,09	0,29 $\pm$ 0,00	0,52 $\pm$ 0,21	0,94 $\pm$ 0,00	0,96 $\pm$ 0,00	1,47 $\pm$ 0,00	1,66 $\pm$ 0,16
Solling	Picea abies	0	4	4,16	4,17	1,80 $\pm$ 0,00	1,82 $\pm$ 0,00	2,09 $\pm$ 0,00	2,10 $\pm$ 0,00	4,58 $\pm$ 0,00	4,60 $\pm$ 0,00
		2		4,15	4,16	1,85 $\pm$ 0,01	1,92	2,10 $\pm$ 0,00	2,10	4,60 $\pm$ 0,03	4,62
		6		4,15	4,2	1,86 $\pm$ 0,00	1,94 $\pm$ 0,00	2,12 $\pm$ 0,00	2,10 $\pm$ 0,01	4,62 $\pm$ 0,04	4,69 $\pm$ 0,05
		13		4,18	4,24	1,89 $\pm$ 0,02	1,94 $\pm$ 0,01	2,12 $\pm$ 0,05	2,07 $\pm$ 0,00	4,76 $\pm$ 0,03	4,58 $\pm$ 0,08
		27		4,19	4,27	1,90 $\pm$ 0,01	2,02 $\pm$ 0,01	2,13 $\pm$ 0,01	2,13 $\pm$ 0,00	4,61 $\pm$ 0,05	4,67 $\pm$ 0,01
		85		4,18	4,29	1,97 $\pm$ 0,02	2,23 $\pm$ 0,01	2,11 $\pm$ 0,01	2,13 $\pm$ 0,02	4,61 $\pm$ 0,04	4,89 $\pm$ 0,04
		0	8	4,16	4,17	1,80 $\pm$ 0,00	1,82 $\pm$ 0,00	2,09 $\pm$ 0,00	2,10 $\pm$ 0,00	4,58 $\pm$ 0,00	4,60 $\pm$ 0,00
		2		4,1	4,2	1,91 $\pm$ 0,02	1,93	2,08 $\pm$ 0,00	2,14	4,66 $\pm$ 0,01	4,74
		6		4,17	4,28	1,87 $\pm$ 0,00	2,02 $\pm$ 0,00	2,13 $\pm$ 0,01	2,10 $\pm$ 0,00	4,71 $\pm$ 0,01	4,82 $\pm$ 0,02
		13		4,1	4,27	1,83 $\pm$ 0,00	2,03 $\pm$ 0,02	2,08 $\pm$ 0,02	2,05 $\pm$ 0,01	4,65 $\pm$ 0,01	4,65 $\pm$ 0,14

Solling	Picea abies	27		4,19	4,37	1,92 ± 0,01	2,22 ± 0,02	2,11 ± 0,01	2,13 ± 0,01	4,56 ± 0,05	4,80 ± 0,09
		85		4,26	4,57	2,09 ± 0,02	2,57 ± 0,05	2,12 ± 0,02	2,14 ± 0,02	4,53 ± 0,05	5,03 ± 0,07
		0	20	4,16	4,17	1,80 ± 0,00	1,82 ± 0,00	2,09 ± 0,00	2,10 ± 0,00	4,58 ± 0,00	4,60 ± 0,00
		2		4,12	4,15	1,89 ± 0,01	1,98	2,10 ± 0,00	2,12	4,73 ± 0,06	4,77
		6		4,15	4,31	1,82 ± 0,00	1,96 ± 0,02	2,13 ± 0,01	2,12 ± 0,01	4,68 ± 0,02	4,69 ± 0,02
		13		4,15	4,32	1,81 ± 0,03	2,03 ± 0,04	2,06 ± 0,01	2,09 ± 0,01	4,47 ± 0,18	4,65 ± 0,10
		27		4,21	4,47	1,90	2,19 ± 0,09	2,10	2,10 ± 0,04	4,44	4,61 ± 0,16
		85		4,26	4,63	2,05 ± 0,03	2,56 ± 0,05	2,14 ± 0,02	2,12 ± 0,02	4,47 ± 0,06	4,96 ± 0,06
		0	30	4,16	4,17	1,80 ± 0,00	1,82 ± 0,00	2,09 ± 0,00	2,10 ± 0,00	4,58 ± 0,00	4,60 ± 0,00
		2		4,16	4,19	1,89 ± 0,00	1,98	2,09 ± 0,01	2,12	4,68 ± 0,03	4,73
		6		4,14	4,28	1,86 ± 0,00	2,07 ± 0,01	2,14 ± 0,00	2,12 ± 0,01	4,70 ± 0,01	4,76 ± 0,01
		13		4,23	4,35	1,82 ± 0,05	2,14 ± 0,00	2,06 ± 0,00	2,07 ± 0,00	4,47 ± 0,16	4,75 ± 0,08
		27		4,22	4,37	1,91	2,17 ± 0,05	2,10	2,15 ± 0,01	4,35	4,55 ± 0,01
		85		4,22	4,66	1,83 ± 0,10	2,19 ± 0,02	2,15 ± 0,01	2,14 ± 0,03	4,37 ± 0,10	4,64 ± 0,00
Lüss	Fagus sylvatica	0	4	5,62	5,72	0,29 ± 0,00	0,28 ± 0,00	0,49 ± 0,00	0,50 ± 0,00	1,2 ± 0,00	1,19 ± 0,00
		2		5,61	5,56	0,30 ± 0,02	0,34	0,5 ± 0,00	0,52	1,15 ± 0,01	1,23
		6		5,43	5,38	0,31 ± 0,02	0,35 ± 0,01	0,50 ± 0,01	0,51 ± 0,01	1,22 ± 0,01	1,21 ± 0,03
		13		5,68	5,7	0,29 ± 0,02	0,37 ± 0,01	0,49 ± 0,01	0,50 ± 0,01	1,23 ± 0,02	1,12 ± 0,21
		27		5,78	5,98	0,25 ± 0,00	0,41 ± 0,02	0,50 ± 0,01	0,52 ± 0,00	1,11 ± 0,02	1,18 ± 0,07
		85		5,5	5,86	0,34 ± 0,01	0,65 ± 0,01	0,50 ± 0,01	0,51 ± 0,01	1,15 ± 0,00	1,46 ± 0,04
		0	8	5,62	5,72	0,29 ± 0,00	0,28 ± 0,00	0,49 ± 0,00	0,51 ± 0,00	1,20 ± 0,00	1,20 ± 0,00
		2		5,39	5,44	0,33 ± 0,01	0,34	0,50 ± 0,00	0,54	1,20 ± 0,03	1,25
		6		5,46	5,59	0,28 ± 0,01	0,35 ± 0,02	0,50 ± 0,00	0,53 ± 0,01	1,23 ± 0,02	1,26 ± 0,01
		13		5,61	5,7	0,22 ± 0,01	0,42 ± 0,08	0,50 ± 0,01	0,50 ± 0,01	1,19 ± 0,01	1,16 ± 0,28
		27		5,98	6,15	0,28 ± 0,01	0,58 ± 0,12	0,50 ± 0,01	0,52 ± 0,01	1,08 ± 0,02	1,32 ± 0,12
		85		6,35	6,57	0,30 ± 0,03	0,83 ± 0,13	0,51 ± 0,01	0,51 ± 0,01	0,99 ± 0,05	1,60 ± 0,12
		0	20	5,62	5,72	0,29 ± 0,00	0,28	0,49 ± 0,00	0,51 ± 0,00	1,20 ± 0,00	1,20 ± 0,00
		2		5,48	5,62	0,32 ± 0,01	0,33	0,51 ± 0,01	0,54	1,23 ± 0,04	1,23
		6		5,42	5,58	0,23 ± 0,02	0,37	0,50 ± 0,00	0,45 ± 0,04	1,15 ± 0,03	1,48 ± 0,42
		13		5,57	5,9	0,07 ± 0,10	0,52	0,49 ± 0,00	0,39 ± 0,11	0,96 ± 0,11	1,64 ± 0,33
		27		5,64	6,27		0,65	0,50 ± 0,00	0,38 ± 0,14	0,83 ± 0,04	1,75 ± 0,62
		85		5,48	5,9		0,81	0,49 ± 0,01	0,48	0,73 ± 0,06	2,08 ± 0,76
		0	30	5,62	5,72	0,29	0,28	0,49 ± 0,00	0,51 ± 0,00	1,20 ± 0,00	1,20 ± 0,00
		2		5,36	5,51	0,31	0,35	0,51 ± 0,01	0,53	1,21 ± 0,01	1,20
		6		5,23	5,43		0,38	0,51 ± 0,01	0,43 ± 0,13	0,89 ± 0,03	1,20 ± 0,37
		13		5,6	5,73		0,47	0,39 ± 0,04	0,39 ± 0,16	0,64 ± 0,07	1,15 ± 0,30
		27		6	6,12		0,53	0,27 ± 0,04	0,39 ± 0,20	0,57 ± 0,01	1,07 ± 0,54
		85		5,7	5,6		0,65	0,10 ± 0,13	0,37 ± 0,18	0,40 ± 0,13	1,25 ± 0,54

Table S3. Frequency of collection of BD/TF water, sampling dates, and details on water filtering. Samples were stored at 2 or 4 °C in the dark between water collection and filtering. While water collected weekly or biweekly was pooled for a given month for assessing nitrogen deposition, for isotopes we used the water collected at each sampling date, over the time window July-October 2016.

Site	Sampling frequency	Sampling 1		Sampling 2		Sampling 3		Filtering for isotopic/genetic analysis
		Start	End	Start	End	Start	End	
Höka	Monthly	01/08/2016	29/08/2016	29/08/2016	03/10/2016	03/10/2016	31/10/2016	Between 1 and 4 weeks from water collection
Thetford	Monthly	11/07/2016	31/07/2016	01/08/2016	28/08/2016	05/09/2016	25/09/2016	Between 1 and 4 weeks from water collection
Brasschaat ¹	Weekly	19/07/2016	11/08/2016	06/10/2016	20/10/2016	N/A	N/A	Between 2 and 4 weeks from water collection
Rogate	Monthly	11/07/2016	01/08/2016	08/08/2016	05/09/2016	05/09/2016	05/10/2016	Between 1 and 4 weeks from water collection
Alice Holt	Monthly	04/07/2016	25/07/2016	01/08/2016	12/09/2016	26/09/2016	10/10/2016	Between 1 and 4 weeks from water collection
Hoeilaart ¹	Weekly	20/07/2016	12/08/2016	07/10/2016	21/10/2016	N/A	N/A	Between 2 and 4 weeks from water collection
Haguenau	Weekly	10/08/2016	07/09/2016	07/09/2016	21/09/2016	21/09/2016	05/10/2016	Between March and May 2017
Schänis	Biweekly	02/08/2016	16/08/2016	16/08/2016	30/08/2016	30/08/2016	13/09/2016	1 day to 1 week from water collection
Bertiz	Monthly ¹	30/06/2016	03/08/2016	03/08/2016	03/10/2016	03/10/2016	24/10/2016	1 day to 1 week from water collection
Collelongo	Weekly	31/08/2016	07/09/2016	07/09/2016	14/09/2016	14/09/2016	19/09/2016	Between October 2016 and February 2017

¹ These sites had no rain in August, so water collected in July was also included.

Table S4. Sequences of oligonucleotides used in the quantification of functional genes involved in main nitrogen processes. For more details on methodological information please refer to Supplementary Text S2.

Target gene	Primers	Sequence	Reference
nifH	nifHF	AAAGGYGGWATCGGYAARTCCACCAC	[25]
	nifHR	TTGTTSGCSGCRTACATSGCCATCAT	
archeal amoA	amo19F	ATGGTCTGGCTWAGACG	[27]
	amo643R	TCCCACTTWGACCARGCGGCCATCCA	[32]
bacterial amoA	amoA1F	GGGGTTTCTACTGGTGGT	[26]
	amoA1R	CCCCTCKGSAAAGCCTTCTTC	
Nitrobacter nxrB	nxrB1F	ACGTGGAGACCAAGCCGGG	[29]
	nxrB1R	CCGTGCTGTTGAYCTCGTTGA	
nirK	nirK876C	ATYGGCGGVCA YGGCGA	[33]
	nirK1040	GCCTCGATCAGRTTRTG GTT	

Figure S1. Differences between throughfall (TF) and bulk deposition (BD) for the abundance of functional genes involved in nitrification across the 10 sites where the TF and BD water were collected (see Methods and Extended Data Table 1). The quantified functional genes were the archaeal and bacterial *amoA* genes (*amoA* AOA and *amoA* AOB, respectively) and the bacterial *nxB* gene. The data for the functional genes were log10-transformed, so the differences between TF and BD for a given gene abundance, and hence the values reported on the y-axis, were obtained as: $\log_{10}(\text{gene abundance})_{\text{TF}} - \log_{10}(\text{gene abundance})_{\text{BD}} = \log_{10}^{\text{TF/BD}}(\text{gene abundance})$. Means and 95% confidence intervals are in black (n=17, n=23 and n=23 for *amoA* AOA, *amoA* AOB and *nxB*, respectively).

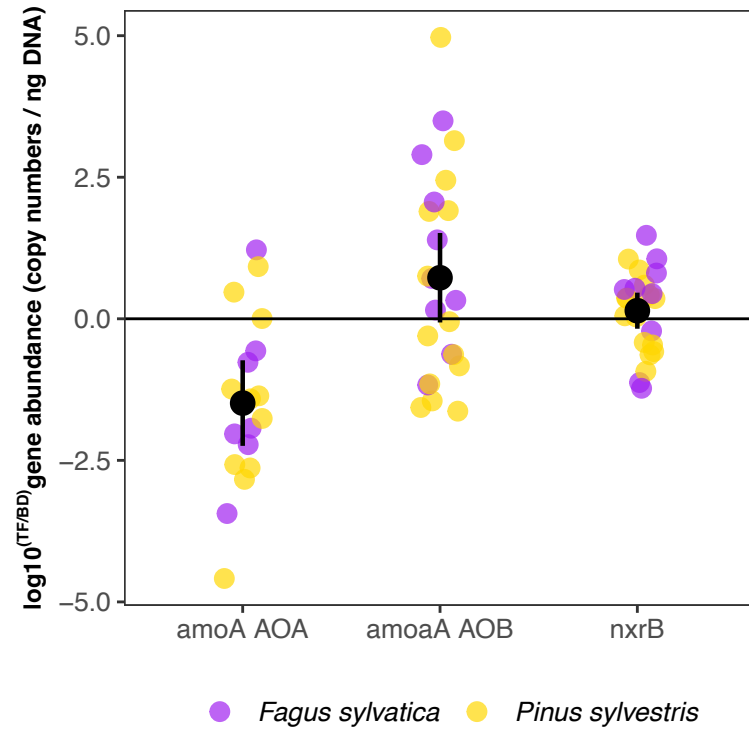


Figure S2. Abundance of the microbial functional genes involved in nitrogen fixation and denitrification. Abundance of bacterial genes that encode for nitrogenase reductase (nifH, panel A) and nitrite reductase (nirK, panel B) involved in nitrogen fixation and the first step of denitrification ($\text{NO}_3^- \rightarrow \text{N}_2\text{O}$), respectively, in the phyllosphere of all forests including the three sites in Finland. Functional genes are expressed relative to nanograms of extracted DNA on the y-axis. The sites on the x-axis follow a latitudinal order from north to south. Number of observations (n) per site is n=3 for both functional genes, except for nifH at Sevettijarvi (n=2) and nirK at Bertiz, Alice Holt, Hoeilaart, Schanis and Collelongo (n=2).

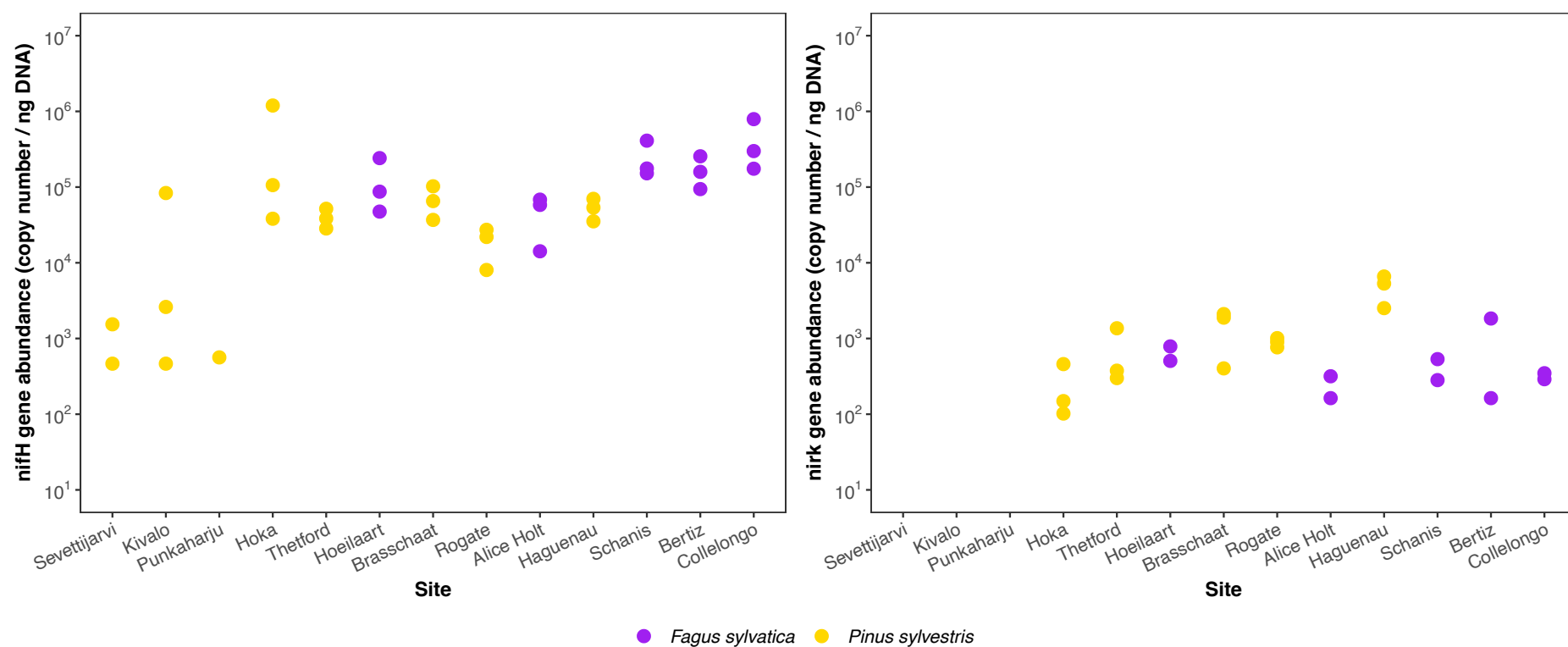


Figure S3. Concentrations of NH_4^+ -N, NO_3^- -N, and TN in the filtered and unfiltered throughfall (TF) water collected the day after a rain in various sites in Germany. After collection, the TF water was subjected to different temperatures (4, 8, 20, and 30 °C) for up to 85 days from sample collection to simulate field and storage temperature conditions (i.e., 2-4 °C). Each point is the average calculated over 2 replicates, but standard deviation is not included for the sake of clarity (see Table S2). Blue dashed lines indicate the sampling frequency at our sites, going from 1 week, 2 weeks or 1 month. The experiment is described in Text S1. Note the different scale used for improving the readability.

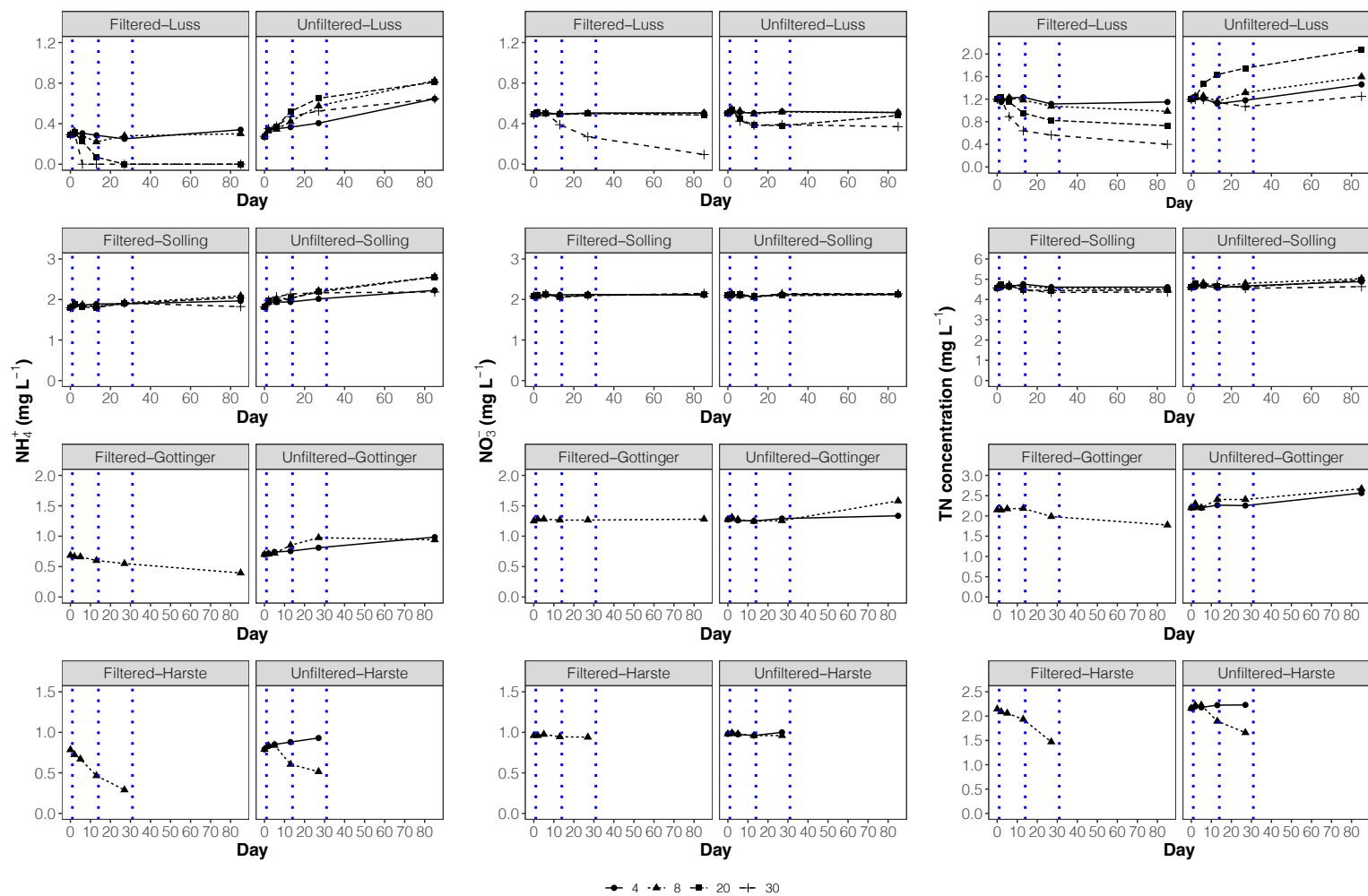
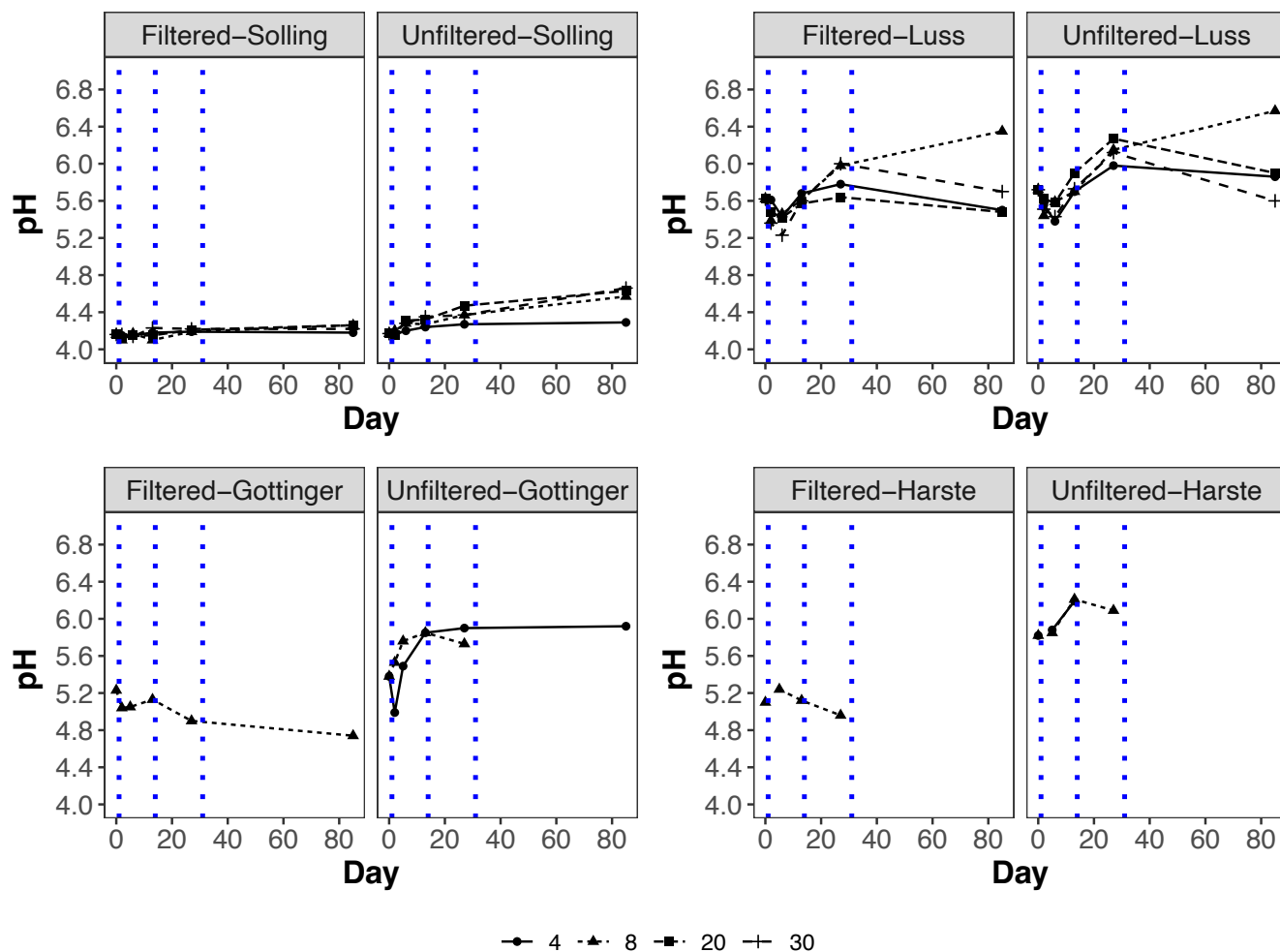


Figure S4. Changes in pH in the filtered and unfiltered throughfall (TF) water collected the day after a rain at four sites in Germany used to monitor changes in the nitrogenous compounds (see Figure S3). After collection, the TF water was subjected to different temperatures (4, 8, 20, and 30 °C) for up to 85 days from sample collection to simulate field and storage temperature conditions (i.e., 2–4 °C). Each point is the average calculated over 2 replicates, but standard deviation is not included for the sake of clarity. Blue dashed lines indicate the sampling frequency at our sites, going from 1 week, 2 weeks or 1 month. The experiment is described in Text S1.



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