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Full ^{15}N tracer accounting to revisit major assumptions of ^{15}N isotope pool dilution approaches for gross nitrogen mineralization

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ABSTRACT

The ^{15}N isotope pool dilution (IPD) technique is the only available method for measuring gross ammonium (NH_4^+) production and consumption rates. Rapid consumption of the added ^{15}N-NH_4^+ tracer is commonly observed, but the processes responsible for this consumption are not well understood. The primary objectives of this study were to determine the relative roles of biotic and abiotic processes in ^{15}N-NH_4^+ consumption and to investigate the validity of one of the main assumptions of IPD experiments, i.e., that no reflux of the consumed ^{15}N tracer occurs during the course of the experiments. We added a ^{15}N-NH_4^+ tracer to live and sterile (autoclaved) soil using mineral topsoil from a beech forest and a grassland in Austria that differed in NH_4^+ concentrations and NH_4^+ consumption kinetics. We quantified both biotic tracer consumption (i.e. changes in the concentrations and ^{15}N enrichments of NH_4^+, dissolved organic N (DON), NO_3^- and the microbial N pool) and abiotic tracer consumption (i.e., fixation by clay and/or humic substances). We achieved full recovery of the ^{15}N tracer in both soils over the course of the 48 h incubation. For the forest soil, we found no rapid consumption of the ^{15}N tracer, and the majority of tracer (78%) remained unconsumed at the end of the incubation period. In contrast, the grassland soil showed rapid ^{15}N-NH_4^+ consumption immediately after tracer addition, which was largely due to both abiotic fixation (24%) and biotic processes, largely uptake by soil microbes (10%) and nitrification (13%). We found no evidence for reflux of ^{15}N-NH_4^+ over the 48 h incubation period in either soil. Our study therefore shows that ^{15}N tracer reflux during IPD experiments is negligible for incubation times of up to 48 h, even when rapid NH_4^+ consumption occurs. Such experiments are thus robust to the assumption that immobilized labeled N is not re–mobilized during the experimental period and does not impact calculations of gross N mineralization.

1. Introduction

Nitrogen (N), in its inorganic forms ammonium (NH_4^+) and nitrate (NO_3^-), is often considered to be the limiting nutrient for plants in terrestrial ecosystems (Falkowski et al., 2008). Primary production, nitrification and denitrification are controlled by the rates at which inorganic N is both produced *via* mineralization of organic N and biological N fixation and consumed by biotic and abiotic processes. The understanding of this continuous cycling between organic and inorganic nitrogen forms is therefore of fundamental importance for estimating plant-available N in agricultural and natural soil systems (Hadas et al., 1992; Vitousek et al., 2002; Ward, 2012). A powerful tool for the determination of soil N transformation processes is the isotope pool dilution (IPD) technique (Barraclough, 1991; Di et al., 2000; Kirkham and Bartholomew, 1954; Wanek et al., 2010), which allows to estimate both rates of gross production and gross consumption of major plant nutrients in soil. This technique has been used across a wide range of natural and agricultural systems to study N transformation rates in soil (e.g., Booth et al., 2005, 2006; Hart et al., 1994; Murphy et al., 2003), and is particularly recognized as the recommended method to obtain estimates on soil N dynamics (Hart et al., 1994). Depending on tracer application approaches e.g. to intact soil-plant systems in situ or to sieved soils, plant mediated processes are included such as root uptake of inorganic N or tracer dynamics only reflect microbial processes such as in sieved soils (Murphy et al., 2003; Rütting et al., 2011).

The IPD approach relies on labeling the target pool, i.e. the product pool of the reaction to be measured, which in the case of N mineralization is the NH_4^+ pool, with ^{15}N-enriched tracer (^{15}N-NH_4^+). The

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isotopic tracer is then diluted as a consequence of mineralization of unlabeled organic N to NH_4^+. Gross N mineralization (i.e., NH_4^+ production or influx) and gross NH_4^+ consumption (i.e., NH_4^+ efflux) are then calculated from the change in size of the total NH_4^+ pool ($^{14}N + {}^{15}N$), and from the decline in the ^{15}N enrichment above natural abundance over time (Barraclough, 1991; Hart et al., 1994; Kirkham and Bartholomew, 1954; Murphy et al., 2003). Kirkham and Bartholomew (1954) stated that the following assumptions need to be met in order to convert the measured quantities and isotope ratios to absolute rates: (i) the isotopically heavy (tracer) and the lighter molecules (tracee) behave in the same way in a soil; (ii) mineralization and immobilization rates remain constant during the interval between successive measurements; (iii) the ratio of tracer to tracee in the efflux is in proportion to that of the labeled pool, and (iv) immobilized labeled N is not remobilized during the experimental period.

The last of these key assumptions – no recycling of tracer consumed during the experiment – could be violated during IPD experiments if rapid consumption of the tracer takes place. It is well known that such a reflux of ^{15}N tracer into the NH_4^+ pool could lead to a substantial error in calculations, resulting in an underestimation of gross mineralization and consumption rates (Barraclough and Puri, 1995; Bjarnason, 1988; Davidson et al., 1991). Rapid consumption of ^{15}N-NH_4^+ has been reported by several studies (e.g. > 50% tracer loss within minutes), but it is not clear which consumption processes are involved or whether remobilization of the ^{15}N tracer is likely (Davidson et al., 1991; Kowalenko and Cameron, 1978; Morier et al., 2008). We here define all processes removing NH_4^+ from the available NH_4^+ pool as consumption processes, following the accepted terminology (Booth et al., 2005; Murphy et al., 2003), which can be further distinguished into biotic NH_4^+ consumption (i.e., microbial uptake and nitrification; hereafter "immobilization") and abiotic NH_4^+ consumption (i.e., fixation by the mineral or organic soil fraction; hereafter "fixation"). Biotic processes are often assumed to be the dominant consumptive processes in IPD experiments lasting for a few days (Monaghan and Barraclough, 1995; Morier et al., 2008; Trehan, 1996). Indeed, several authors have reported microbial uptake of inorganic and organic compounds within minutes and even seconds after tracer addition (Farrell et al., 2011; Hill et al., 2012; Jones et al., 2013; Tahovská et al., 2013; Wilkinson et al., 2014). Nevertheless, others have suggested abiotic fixation to be the main mechanism explaining rapid NH_4^+ consumption (Davidson et al., 1991; Johnson et al., 2000; Trehan, 1996). In fact, NH_4^+ fixation by clay minerals is known to occur within h after NH_4^+ addition (Cavalli et al., 2015; Nieder et al., 2011; Nõmmik and Vahtras, 1982). Physical sorption or chemisorption to organic matter might also be responsible for the removal of ^{15}N-NH_4^+ from the extractable N pool (Mortland and Wolcott, 1965; Nieder et al., 2011; Nõmmik and Vahtras, 1982). However, despite the potential for biotic and abiotic processes to rapidly consume ^{15}N-NH_4^+ during IPD experiments, the sinks involved have not as yet been clearly quantified.

The objective of this study was to determine the fate of added ^{15}N-NH_4^+ during the duration that ^{15}N-IPD experiments usually last (i.e., < 48 h) in two sieved soils that differ in their NH_4^+ consumption rates. We considered all possible sources of tracer reflux to evaluate whether the requirement that consumed labeled N is not remobilized during the experimental duration of normal IPD experiments is valid. Additionally we investigated the constancy of transformation rates over time. We hypothesized that rapidly consumed ^{15}N tracer is mainly subjected to biotic (microbial) immobilization processes, that the ^{15}N tracer can therefore be remineralized or released during the incubation period, and that such reflux causes an underestimation of gross N mineralization fluxes in soils that exhibit rapid ^{15}N-NH_4^+ consumption.

Table 1
Selected soil characteristics of the top soil (0–10 cm) of the forest and the grassland soil (means ± 1 SE, n = 3).

Soil parameter		Forest	Grassland
Soil pH		4.0 ± 0.0	6.0 ± 0.0
Soil texture	Clay (%)	16.3 ± 0.1	26.2 ± 1.3
	Silt (%)	63.4 ± 0.5	56.1 ± 1.7
	Sand (%)	20.3 ± 0.6	17.7 ± 1.0
Soil C & N content	Total C (%)	3.4 ± 0.7	2.9 ± 0.5
	Total N (%)	0.2 ± 0.0	0.3 ± 0.1
Soil C:N ratio		13.4 ± 0.2	10.1 ± 0.2
Soil NH_4^+ concentration	(μg N g^{-1} d.w.)	29.2 ± 0.7	1.3 ± 0.1
^{15}N-NH_4^+ tracer recovery	(%; after 15 min)	99 ± 0.2	41 ± 2.8

2. Materials and methods

2.1. Sampling site and soil description

Soils were collected from two sites in Austria differing in vegetation composition and soil pH (Table 1). Top soils were sampled from a beech (*Fagus sylvatica* L.) forest (N 48.228656°, E 16.260713°, 382 m a.s.l., Schottenwald, Vienna) and from a permanent grassland (N 48.049063°, E 16.197592°, 323 m a.s.l., Mödling, Lower Austria). The soils are hereafter referred to as "forest" and "grassland" soil, respectively. The forest soil is classified as a dystric Cambisol (Kaiser et al., 2010) and the grassland soil as a Cambisol (Nestroy et al., 2011). Samples were taken from the upper 10 cm of the mineral soil (A) horizon in October 2014. The soil was sieved to 2 mm and stored at 4°C until experiments were performed. Soil pH was measured in 10 mM $CaCl_2$. Total carbon (C) and N contents were measured in finely ground, oven dried (105°C, 24 h) soil using an elemental analyzer (EA1110, CE Instruments, Milan, IT) coupled to a continuous flow stable isotope ratio mass spectrometer (DeltaPLUS, Thermo Finnigan, Bremen, DE) (EA–IRMS). Soil ammonium contents were determined photometrically in 1 M KCl extracts [soil to extractant ratio of 1:7.5 (w:v)] based on the Berthelot reaction (Hood-Nowotny et al., 2010). Soil texture analysis was done based on a micropipette method modified from Miller and Miller (1987), by using 5% sodium hexametaphosphate as a dispergent.

The soils were selected because of their similarity in general soil properties, such as soil texture (silt loam) and C and N content, but they differed considerably in soil pH and available NH_4^+ content (Table 1). Additionally, the soils strongly differed in their consumption of added $^{15}NH_4^+$ as determined in a preliminary tracer recovery experiment, in which both soils were labeled with 10 atom% $(^{15}NH_4^+)_2SO_4$ solution (20% of the initial NH_4^+ pool) and after 15 min extracted with 0.5 M K_2SO_4 [soil to extractant ratio of 1:7.5 (w:v)]. We found that 99% of the added ^{15}N tracer could be recovered as NH_4^+ from the forest soil but only 41% from the grassland soil (Table 1).

2.2. Experimental design

The IPD assay was performed with two treatments, live (non-sterilized) and sterilized (autoclaved) soil, to distinguish between biotic immobilization processes and abiotic fixation (Fig. 1). Five consecutive measurements of concentrations and isotopic composition of NH_4^+, NO_3^-, microbial biomass N (N_{mic}), and dissolved organic N (DON) were taken over the course of 48 h. To obtain high-resolution time kinetics of measured processes, we stopped incubations within 2–3 min (0 h), 0.25 h, 3.5 h, 24 h, and 48 h after tracer addition. We thereby accommodated the standard experimental duration suggested by Murphy et al. (2003) (i.e. t_1: 4 h–24 h; t_2 48 h–144 h), with two additional early sampling points to track rapid consumptive processes. In addition, the contribution of abiotic fixation (i.e., fixation by clay and humic substances) was determined at two fixed time points (0 h and 24 h) in live soils (Fig. 1).

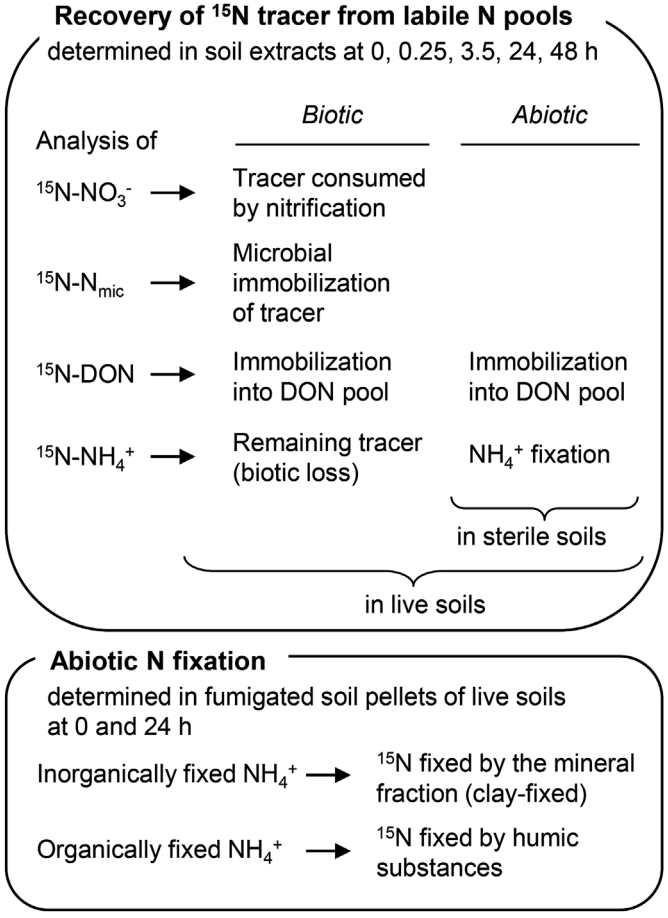

Fig. 1. Overview of experimental design of the isotope pool dilution experiment. The IPD assay was performed with two treatments, live (non–sterilized) and sterilized (autoclaved) soil, to distinguish between biotic immobilization processes and abiotic fixation. Five consecutive measurements of concentrations and isotopic composition of NH_4^+, NO_3^-, microbial biomass N (N_{mic}), and dissolved organic N (DON) were taken over the course of 48 h. To obtain high-resolution time kinetics of measured processes, we stopped incubations immediately (0 h), 0.25 h, 3.5 h, 24 h, and 48 h after label addition. In addition, the contribution of abiotic fixation (i.e., fixation by clay and humic substances) was determined at two fixed time points (0 h and 24 h).

2.3. Soil sample preparation and sterilization procedure

We adjusted the soils to approximately 50% water holding capacity (WHC) prior to the IPD experiment. Following this, 4 g of fresh soil was weighed into 50 mL glass vials (Crimp Top Headspace Vials, Supelco, US) and covered with Parafilm (live soils) and aluminum foil (soils to be autoclaved). The soils were prepared in triplicates for each time period, treatment (control or autoclaving) and extraction method (± chloroform). Part of the soil samples were sterilized by autoclaving twice at 121°C for 20 min (Wolf et al., 1989). Between the two autoclaving cycles, samples were incubated at 20°C for 2 days, to allow spores to germinate prior to the second autoclaving cycle. Two hours passed between the second autoclaving cycle and the start of the tracer experiment during which samples were allowed to cool to room temperature and the water content was checked gravimetrically.

2.4. Isotope pool dilution experiment

Shortly before the experiment, soil NH_4^+ contents were determined in soil extracts [1 M KCl, soil to extractant ratio of 1:7.5 (w:v)], based on the Berthelot reaction (Hood-Nowotny et al., 2010). A maximum of 20% of the initial NH_4^+ pool of live soils was added as ^{15}N-NH_4^+ tracer solution at 10 atom%. This approach increased the product pool as little as possible (thus avoiding stimulation of microbial NH_4^+ immobilization processes) whilst also ensuring sufficient enrichment of the NH_4^+ pool with ^{15}N-NH_4^+ to facilitate high measurement precision (Davidson et al., 1991; Di et al., 2000). We applied 400 µL tracer solution (0.5 mM and 0.1 mM $(^{15}NH_4)_2SO_4$ for the forest and grassland soil, respectively) to each sample (4 g fresh weight) in multiple drops across the soil surface and mixed by shaking to achieve homogeneous labeling and a SWC of 70% WHC. The samples were then incubated at 20°C in the darkness for the given incubation periods.

The incubations were stopped by extraction with 30 mL 0.5 M K_2SO_4 solution. The vials were capped with air tight butyl septa and crimp seals (Supelco, US), and shaken horizontally for 30 min at 150 rpm on an orbital shaker. Following extraction, all soil suspensions were gravity filtered through ashless Whatman filter papers. Filters were pre–rinsed with 0.5 M K_2SO_4 and deionized water and dried in a drying oven at 60°C to avoid the variable NH_4^+ contamination from the filter paper. All soil extracts and the extracted soil residues remaining in the filters (see below, determination of fixed N) were stored at −20°C for further analysis.

2.5. Determination of isotope ratios and concentrations of NH_4^+, NO_3^-, DON and microbial biomass N

Filtered extracts were analyzed for the concentration and isotopic composition of NH_4^+ to calculate gross mineralization and consumption rates, and to estimate the recovery of added $^{15}NH_4^+$ over time. We prepared the extracts for isotope ratio mass spectrometry using a microdiffusion approach following Lachouani et al. (2010). Briefly, 10 mL aliquots of samples were diffused with 100 mg magnesium oxide (MgO) into teflon-coated acid traps for 48 h on an orbital shaker. The traps were dried and subjected to EA–IRMS for ^{15}N:^{14}N analysis of NH_4^+.

Concentrations and N isotope ratios of NO_3^- in extracts were determined using a method that is based on the conversion of NO_3^- to NO_2^- with vanadium (III) chloride (VCl_3) and reduction of NO_2^- to N_2O by sodium azide (Lachouani et al., 2010). Concentrations and N isotope ratios of the resulting N_2O were determined by purge–and–trap isotope ratio mass spectrometry (PT–IRMS), using a Gasbench II headspace analyzer (Thermo Fisher, Bremen, DE) with a cryo–focusing unit, coupled to a Finnigan Delta V Advantage IRMS (Thermo Fisher, Bremen, DE).

Concentrations of DON were calculated from the difference between total dissolved N (TDN) and inorganic N (i.e., NH_4^+ and NO_3^-), and the N isotope ratio of DON was calculated using an isotopic mass balance equation (Fry, 2006). Determination of TDN was carried out by conversion of DON and NH_4^+ to NO_3^- by alkaline persulfate oxidation (Cabrera and Beare, 1993; Doyle et al., 2004; Lachouani et al., 2010) and subsequent measurement of formed NO_3^- by the VCl_3-azide method *via* PT-IRMS as described above. Complete conversion of DON to NO_3^- was validated by the parallel digestion of ^{15}N labeled glycine standards (at different atom% ^{15}N), along with unlabeled glycine standards at different concentrations and blanks (Lachouani et al., 2010).

For determination of microbial biomass N (N_{mic}) we performed a simultaneous chloroform fumigation extraction (sCFE) method modified from Setia et al. (2012), thus avoiding relatively long fumigation periods used in the traditional CFE method (Brookes et al., 1985; Tate et al., 1988). For sCFE we carried out parallel soil labeling experiments and performed extractions with 30 mL 0.5 M K_2SO_4 solution amended with 0.5 mL of EtOH-free $CHCl_3$. N_{mic} was calculated from the difference between TDN extracted by 0.5 M K_2SO_4 with and without addition of liquid chloroform (Setia et al., 2012) and its isotope ratio using an isotopic mass balance equation (Fry, 2006). We did not apply a conversion factor (K_{EN}) to correct for non–extractable microbial N, such as N bound in cell walls (Brookes et al., 1985; Jenkinson et al., 2004) since assimilated ^{15}N is supposed to be still in relatively labile forms at least after one day of incubation (Davidson et al., 1991).

2.6. Determination of abiotic fixation in inorganic and organic nitrogen pools

Live soils from the sCFE approach, i.e., pre-extracted with chloroform-amended K_2SO_4 solution can only hold $^{15}NH_4^+$ consumed by abiotic fixation since NH_4^+, NO_3^-, DON and labile N_{mic} has already been extracted. We thus determined the total fixed N (TN_{fixed}) content and the isotopic composition for all live soil samples subjected to sCFE 0 h and 24 h after tracer addition. Frozen, pre-extracted live soil residues were homogenized with a spatula, weighed into 100 mg aliquots and washed with 1.5 mL ultrapure water (shaken for 15 min at 140 rpm) to eliminate any remaining extractant and extractable N. Following centrifugation (1500 × g for 10 min), the supernatant was discarded and the remaining soil was dried at 60 °C for two days, ground, weighed into tin capsules and measured for N content and for N isotopic composition *via* EA–IRMS. We additionally analyzed a set of control soils that received no ^{15}N amendment using the same procedure to correct TN_{fixed} for background ^{15}N levels.

We distinguished between $^{15}N_{fixed}$ held within the clay lattice (i.e., the mineral fraction) and $^{15}N_{fixed}$ held by the soil organic material following a standard extraction procedure for soil organic matter (Stevenson, 1994). Specifically, a second set of soil aliquots (100 mg) was washed with 1.5 mL ultrapure water, centrifuged, the supernatant decanted and 0.5 mL 0.5 M NaOH added to the soil at a ratio of 1:5 (soil:NaOH; Wolf et al., 1994). The soils were then extracted for 18 h (2 h in an ultrasonic bath, 16 h on an orbital shaker at 150 rpm) and centrifuged (1500 × *g* for 10 min). Then a 50 µL aliquot of the supernatant (containing humic compounds) was pipetted into tin capsules, dried (60°C until dry) and measured for N content and N isotopic composition *via* EA–IRMS. Another set of unlabeled soil samples was treated as above and served as ^{15}N natural abundance blanks for calculations. After correcting for soil organic matter extraction efficiency (approximately 80%, Stevenson, 1994), we subtracted the ^{15}N recovery of humic substances from the ^{15}N recovery in TN_{fixed} to obtain the ^{15}N recovery of ^{15}N fixed by the mineral fraction of the soil.

2.7. Data and statistical analyses

In order to investigate the fate of the ^{15}N tracer, we calculated the recovery rate of the added ^{15}N for all N pools as the total amount of ^{15}N recovered divided by the amount added (Hart et al., 1994). These calculations are based on atom percent excess (APE) values calculated for each pool as atom% ^{15}N of the sample minus the natural ^{15}N abundance in unlabeled control samples, and then APE divided by 100 and multiplied by the pool size.

Gross NH_4^+ production (GP; Equation (1)) and gross NH_4^+ consumption (GC; Equation (2)) were calculated for all treatments and time intervals following Kirkham and Bartholomew (1954):

$$GP = \frac{C_{t2} - C_{t1}}{t_2 - t_1} \times \left(\frac{\ln\left(\frac{APE_{t1}}{APE_{t2}}\right)}{\ln\left(\frac{C_{t2}}{C_{t1}}\right)} \right) \quad (1)$$

$$GC = \frac{C_{t1} - C_{t2}}{t_2 - t_1} \times \left(1 + \frac{\ln\left(\frac{APE_{t2}}{APE_{t1}}\right)}{\ln\left(\frac{C_{t2}}{C_{t1}}\right)} \right) \quad (2)$$

Where t_1 and t_2 represent incubation stop times, C_{t1} and C_{t2} represent soil NH_4^+ concentrations (µg N g^{-1} d.w.), and APE is ^{15}N atom% excess.

We used linear models (LMs) to test for effects of sterilization, time, and their interaction on the recovery rates of the added tracer in different pools. Models were validated graphically and, where necessary, refined to account for unequal variance between levels of explanatory variables. We determined the significance of fixed effects using single term deletions combined with likelihood ratio tests (LR) followed by Tukey post-hoc tests. As tracer recovery from the N_{mic} pool was not determined in sterilized soils, we only tested for the effect of time on the recovery rate from the N_{mic} pool. Finally, we performed linear regressions of time against the natural logarithm of APE to investigate the constancy of process rates over time. Statistical analyses were performed in R (R Foundation for Statistical Computing, Vienna, 2011) using the packages "nlme" (Pinheiro et al., 2016) and "MASS" (Venables and Ripley, 2002).

The possible impact of ^{15}N reflux from the N_{mic} pool on gross NH_4^+ production rates was investigated in both soils using sensitivity analysis. Reflux rates of ^{15}N-NH_4^+ of 10%, 20%, 50%, and 100% were simulated for the incubation period from 3.5 h to 24 h, which is considered to be an appropriate incubation time during isotope pool dilution experiments (Murphy et al., 2003). The initial NH_4^+ concentrations and APE at t0 (3.5 h) were kept constant, but NH_4^+ concentrations and APE at t1 (24 h) were recalculated for the different scenarios. We simulated reflux for the amount of ^{15}N-N_{mic}, which was rapidly taken up by microbes during the first 15 min of incubation time. Therefore, the APE of ^{15}N-NH_4^+ for the mean atom% enrichment in the initial incubation phase (APE at t1 = $\bar{x}_{APE}$ at 0 h, 0.25 h) needed to be recalculated. The tracer reflux was calculated at increasing rates of 10%, 20%, 50%, and 100% of ^{15}N-N_{mic}. In order to correct the APE of ^{15}N-NH_4^+ at t_1 for the ^{15}N-N_{mic} reflux, we added the average amount of ^{15}N-N_{mic} in excess at 3.5 h and 24 h to the amount of ^{15}N-NH_4^+ in excess at 24 h and recalculated it back to the APE ^{15}N-NH_4^+. Since a reflux of ^{15}N would be coupled to a reflux of NH_4^+ at natural abundance we corrected the concentration of NH_4^+ at t_1 for that amount. From the average atom% enrichment of the NH_4^+ during the initial incubation phase (0 h–0.25 h), and the average amount of ^{15}N in excess calculated for t_1, we were able to estimate the amount of NH_4^+ feeding back into the available ammonium pool concomitant with the respective amount of ^{15}N-NH_4^+. We subsequently estimated the gross NH_4^+ production rates to assess the importance of an eventual reflux of labeled NH_4^+ taken up by microbes into the available ammonium pool.

3. Results

3.1. Total ^{15}N recovery in labile and fixed N pools

We found complete ^{15}N recovery from live grassland and forest soils in the combined fixed and labile N pool, the labile N pool representing the sum of the extractable N pool (NH_4^+, NO_3^- and DON) plus the microbial N pool (Table 2). In live forest soils we recovered 108% (0 h) and 116% (24 h) in the fixed and labile N pool, while in live grassland soils total recoveries ranged between 111% (0 h) and 103% (24 h). Time had a significant effect on total ^{15}N recoveries in both soils (Table 2) but mean values were indistinguishable from 100%, given the large variance around the mean which arises from the propagation of measurement errors for concentration and atom%^{15}N from six different

Table 2
Contribution of different N pools including labile N pools and abiotic fixation by clay and humic substances as sinks of added ^{15}N-NH_4^+ in live forest and live grassland soils during the IPD experiment at incubation times 0 h and 24 h (%, means ± 1 SE, n = 3). Asterisks indicate a significant difference between the time points 0 h and 24 h for each individual soil (*t*-test).

N pool	Forest			Grassland		
	0 h	24 h		0 h	24 h	
NH_4^+	102 ± 1.5	95.5 ± 0.4	*	61.6 ± 2.5	1.5 ± 0.7	*
NO_3^-	0.3 ± 0.0	4.1 ± 0.0	*	13 ± 0.1	54.9 ± 2.6	*
DON	2.6 ± 0.8	0.3 ± 0.3	*	2 ± 0.6	2.1 ± 0.4	*
N_{mic}	1.6 ± 0.2	0.4 ± 0.4	*	10.3 ± 1.3	6 ± 0.4	*
Clay N_{fixed}	1.7 ± 0.4	15.3 ± 0.9	*	23.9 ± 2.7	37.2 ± 0.4	*
Humic N_{fixed}	0.0 ± 0.0	0.1 ± 0.1		0.1 ± 0.1	1.1 ± 0.3	*
Sum	108 ± 11	116 ± 10	*	111 ± 6	103 ± 6	*

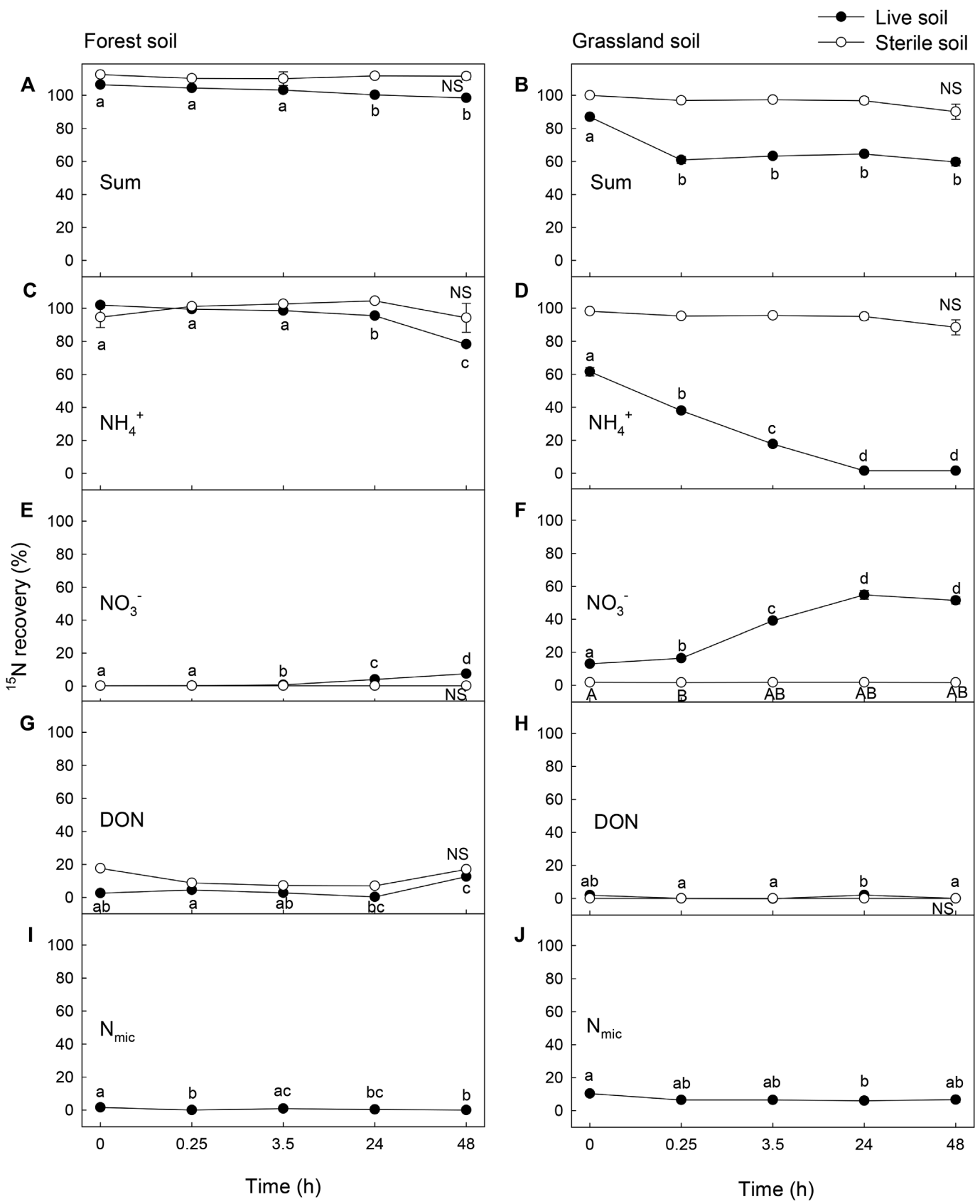

Fig. 2. Mean recovery rates (%, ± 1 SE, $n = 3$) measured in the sum of labile N (A, B), NH_4^+ (C, D), NO_3^- (E, F), DON (G, H) and N_{mic} (I, J) over the incubation time in the live and sterile forest soil (left panel) and the live and sterile grassland soil (right panel). Labile N represents the sum of NH_4^+, NO_3^-, DON, and N_{mic}. Significant differences between time points were tested by linear models and Tukeys HSD post-hoc test and are given by different lower case letters (live soils) or upper case letters (sterile soils); NS, not significant ($P > 0.05$). Error bars fell within the confines of the symbols in some instances.

Table 3
Effect of time (T) and sterilization (S) and significance of interaction between time and sterilization (TxS) on recovery rates of ^{15}N (%) from extractable N pools (NH_4^+, NO_3, DON), the microbial N pool (N_{mic}), and the sum of extractable and microbial N pool (labile N) in the forest soil and the grassland soil. The model did not allow for determining the significance of treatment and of interaction between time and sterilization on the recovery rate of the tracer from the microbial N pool (NA), as recovery was not determined in the sterilized soils. Values are given for the likelihood ratio test (LR), the degrees of freedom (*df*), and the significance level of the individual term or the interaction term on the recovery rate of ^{15}N. Asterisks indicate the significance of a single variable (T, S) or the interaction of variables (TxS) on the recovery rate of ^{15}N (*P < 0.05, **P < 0.01, ***P < 0.001).

N pool	Factor	Forest LR	*df*	*P*	Grassland LR	*df*	*P*
NH_4^+	T	23.1	4,12	< 0.001***	22.0	4,11	< 0.001***
	S	11.7	1,12	< 0.001***	94.3	1,11	< 0.001***
	TxS	15.4	4,16	0.004**	70.4	4,15	< 0.001***
NO_3^-	T	16.3	4,11	0.003**	13.5	4,11	0.009**
	S	2.3	1,11	0.132	120.5	1,11	< 0.001***
	TxS	111.4	4,15	< 0.001***	111.7	4,15	< 0.001***
DON	T	36.2	4,12	< 0.001***	12.0	4,7	0.017*
	S	19.9	1,12	< 0.001***	6.3	1,7	0.012*
	TxS	4.7	4,16	0.321	20.3	4,11	< 0.001***
N_{mic}	T	23.3	4,6	< 0.001***	11.5	4,6	0.021*
	S	NA	NA	NA	NA	NA	NA
	TxS	NA	NA	NA	NA	NA	NA
Sum	T	12.4	4,11	0.015*	11.8	4,11	0.019*
	S	45.8	1,11	< 0.001***	100.3	1,11	< 0.001***
	TxS	18.3	4,15	0.001**	164.6	4,15	< 0.001***

pools that finally make up total ^{15}N recovered.

3.2. ^{15}N recovery in labile N pools

We recovered 99–113% of added ^{15}N tracer from the labile N pool in the sterile and live forest soil over the 48 h incubation period (Fig. 2A and B), being significantly higher in sterile than in live soils and decreasing slightly with time but only in live forest soils (Table 3). In the grassland soil, tracer recoveries in labile N in sterile soil were constant, ranging from an initial 100% at 0 h to 90% after 48 h, whereas in the live grassland soil ^{15}N recoveries in labile N decreased significantly from 87% at 0 h to 60% after 48 h (Fig. 2, Table 3).

In the forest soil, the ^{15}N recovery in the NH_4^+ pool decreased in the live soil by approximately 6% at 24 h and 23% at 48 h while in the sterile soil the recovery only varied non-significantly between 95% and 102% (Fig. 2, Table 3). Concomitantly the tracer recovery in the NO_3^- pool increased from 0.3% (0 h) to 4% at 24 h and to 7.5% after 48 h in live forest soil while in the sterile forest soil the recovery rate remained low between 0.2% and 0.3%. We recovered between 0.3 and 3% in the DON pool of live forest soil and up to 19% in sterile forest soil. Sterilization increased the recovery rate of added ^{15}N in the DON pool, but the time course was similar in sterile and live soil samples (Fig. 2, Table 3). ^{15}N recovery in microbial biomass was not measured in autoclaved soil, and decreased over time from 1.6 to 0.4% in live forest soil.

In the live grassland soil, the ^{15}N recovery in the NH_4^+ pool decreased from 62% at 0 h to 1.5% after 24 h and 48 h, while in the sterile grassland soil recovery rates ranged between 98% (0 h) and 88% (48 h) but did not change significantly with time (Fig. 2, Table 3). In parallel to the decrease in the ^{15}N recovery in the NH_4^+ pool in the live grassland soil the recovery rate of ^{15}N in the NO_3^- pool increased significantly from 13% (0 h) to 52% (48 h). The recovery rates for the NO_3^- pool in the sterilized grassland soil varied at around 1.7% and did not change over time. We did not observe consistent changes in the ^{15}N recovery in DON over incubation time, either in live or in sterile grassland soils, with values ranging between 0 and 2% (Fig. 2, Table 3). ^{15}N recoveries in microbial biomass in live grassland soil declined from 10.3% (0 h) to 6.0–6.6% (24 and 48 h).

3.3. ^{15}N recovery in abiotic fixed N pools

Abiotic N fixation in clay minerals and humic substances was measured in live soils after fumigation and extraction with K_2SO_4. Total N fixation (TN_{fixed}) increased in the forest soil, from 1.7% of added ^{15}N (0 h) to 15.4% (24 h, Table 2). In grassland soil a greater proportion of added $^{15}NH_4^+$ tracer was abiotically fixed, and TN_{fixed} increased from 24% to 38.3% within 24 h (Table 2). Fixed ^{15}N from the NH_4^+ pool was mainly recovered in the inorganic N fraction (clay fixation, > 97%), organic N fixation (humic fixation) contributing less than 3% to total abiotic fixation (Table 2).

3.4. Assessment of $^{15}NH_4^+$ reflux and its effect on gross N mineralization rates

In live soils the amount of ^{15}N recovered in different NH_4^+ sinks either increased significantly over incubation time (Forest soil: NO_3^-, DON, N_{fixed}; Grassland soil: NO_3^-, N_{fixed}) or did not show a clear trend (Grassland soil: DON; Fig. 2). Only in the N_{mic} pool, we found a decrease of the recovery rates of added ^{15}N in both soils, i.e., from 1.6% to 0.4% in the forest soil and from 10.3% to 6% in the grassland soil, while recoveries in N_{fixed} increased rather than decreased (Table 2). Therefore, we identified N_{mic} as the main possible source for reflux of immobilized labeled NH_4^+ to the available ammonium pool over incubation time. The possible impact of ^{15}N reflux from the N_{mic} pool on gross NH_4^+ production rates was investigated in both soils using sensitivity analysis, at reflux rates of 10%, 20%, 50%, and 100% of the ^{15}N-N_{mic} pool for an incubation period from 3.5 h to 24 h. In the forest soil, the reflux caused only a modest underestimation of gross NH_4^+ production rates and at a reflux rate of 10%, NH_4^+ gross production rates were not affected at all (Table 4). In contrast, a simulated worst-case scenario for the grassland soil revealed an underestimation of gross NH_4^+ production rates by up to 63% (reflux rate of 100% of the rapidly consumed $^{15}NH_4^+$ by soil microbes). At a reflux rate of 50%, the rate was still underestimated by 43% and by 14% at a reflux rate of 10% of the labeled NH_4^+ that was rapidly taken up by microbes (Table 4).

3.5. Consistency of NH_4^+ transformation rates

To test for constant rates of isotope pool dilution over time, which causes an exponential decline in ^{15}N:^{14}N ratios, we plotted the natural logarithm of ^{15}N atom percent excess against incubation time. Given constant rates this plot provides a linear relationship, while declines or increases in isotope pool dilution rates cause curvilinearity (Fig. 3). In the forest soil, we found transformation process rates to be constant between 3.5 h and 48 h (R^2 = 0.979) and in the grassland soil between 0.25 h (and 3.5 h) and 24 h of incubation (R^2 = 0.904). The ^{15}N atom percent excess decreased faster in the grassland soil (k = −0.128) as compared to the forest soil (k = −0.003) in the respective incubation periods. Calculating gross NH_4^+ transformation rates for these time

Table 4
Sensitivity analysis of the effect of ^{15}N-NH_4^+ reflux at different rates from the N_{mic} pool (10%, 20%, 50%, and 100%) on the gross N mineralization rate (mean ± 1 SE, *n* = 3) between incubation time 3.5 h and 24 h, simulated for the forest soil and the grassland soil.

^{15}N reflux (%)	Forest soil		Grassland soil	
	N mineralization (μg N g^{-1} d.w. d^{-1})	Difference (%)	N mineralization (μg N g^{-1} d.w. d^{-1})	Difference (%)
0	2.97 ± 0.2		4.07 ± 0.5	
10	2.96 ± 0.2	0	3.51 ± 0.3	−14
20	2.95 ± 0.2	−1	3.11 ± 0.3	−24
50	2.93 ± 0.2	−2	2.30 ± 0.4	−43
100	2.88 ± 0.2	−3	1.51 ± 0.4	−63

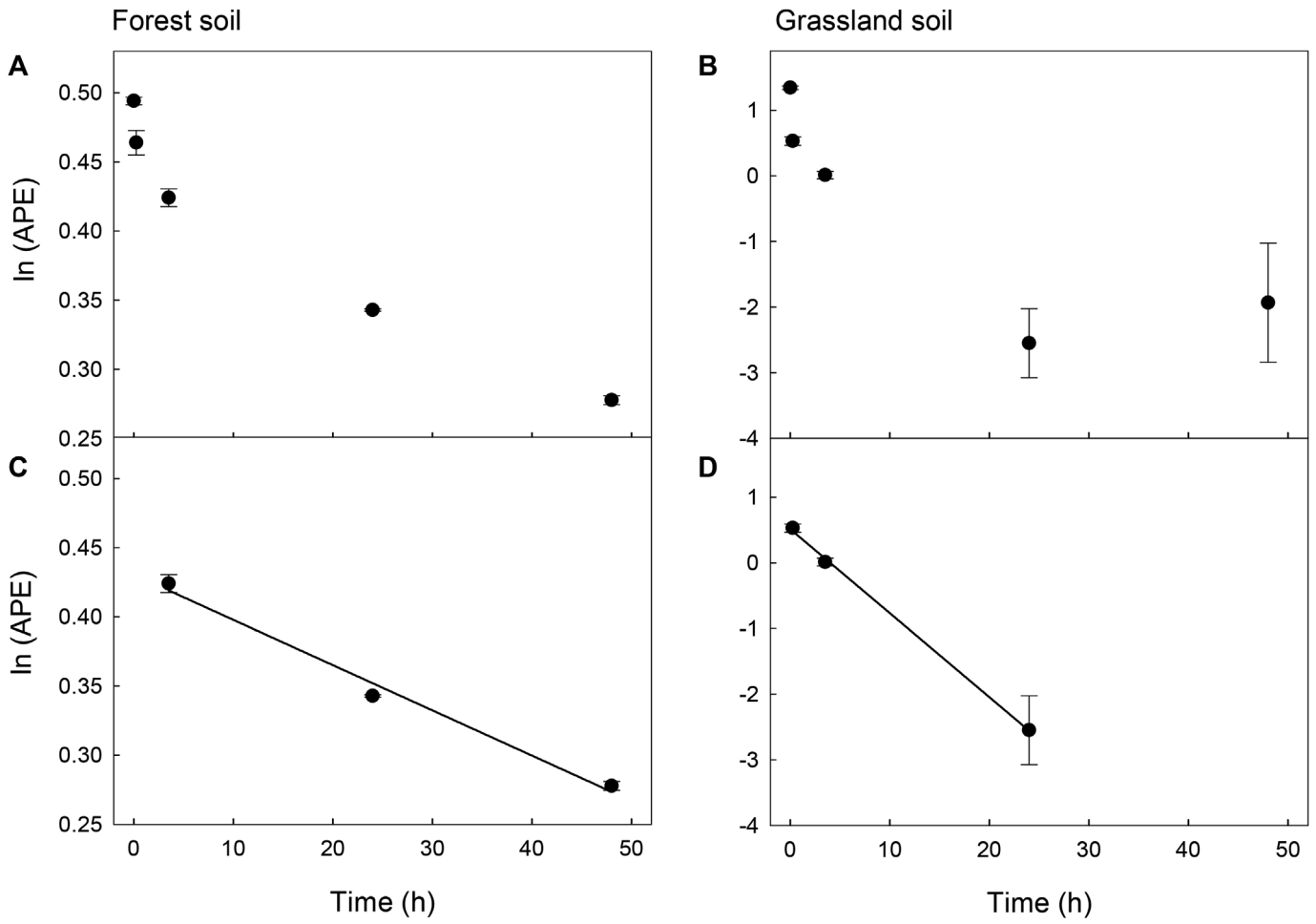

Fig. 3. Change in natural logarithmic atomic percent excess (APE) of $^{15}N\text{-}NH_4^+$ in live forest soils (A) over the total incubation period (0 h–48 h) and (C) over incubation time 3.5 h–48 h, and in live grassland soils (B) over the total incubation period (0 h–48 h) and (D) over incubation time 0.25 h–24 h. Data given are means ± 1SE (n = 3). The linear regressions in (C) and (D) are based on APE estimations at three time points in the forest soil (k = −0.003, R^2 = 0.9786, $P < 0.001$) and in the grassland soil (k = −0.128, R^2 = 0.9044, $P < 0.001$). Error bars fell within the confines of the symbols in some instances.

intervals, we found significantly higher gross N mineralization rates in the grassland soil (5.3 ± 0.1 μg N g^{-1} d.w. d^{-1}) compared to the forest soil (2.3 ± 0.1 μg N g^{-1} d.w. d^{-1}; $P < 0.01$; *t*-test).

4. Discussion

The objectives of this study were to assess the main sink pathways of $^{15}N\text{-}NH_4^+$ tracer during a short-term IPD experiment and to explore whether a reflux of consumed ^{15}N tracer into the available NH_4^+ pool is likely for any of the identified NH_4^+ sinks during incubation time. Such an evaluation is important since a reflux of tracer can significantly impact gross N mineralization rate estimates in soils.

4.1. Recovery of the $^{15}N\text{-}NH_4^+$ tracer

For the live forest soil, we found almost no rapid consumption of ^{15}N tracer and the biggest proportion of the tracer (78% after 48 h) was actually recovered as NH_4^+ at the end of the incubation (Fig. 2). Biological processes (microbial uptake and nitrification) accounted only for a small proportion of $^{15}N\text{-}NH_4^+$ consumed during the incubation. The remaining consumed tracer was recovered in the DON pool (13% after 48 h) and was also found to be abiotically fixed in clay (15% after 24 h). In contrast, the live grassland soil showed rapid $^{15}N\text{-}NH_4^+$ consumption and high NH_4^+ turnover rates, as the tracer in the NH_4^+ pool was depleted by the end of the incubation period, and nitrification was the main consumptive process (Fig. 2). The rapid consumption of $^{15}N\text{-}NH_4^+$ in this soil was striking because nearly half of the tracer was consumed shortly after tracer addition, and was clearly due to both biotic processes (microbial uptake and nitrification; 23%) and abiotic fixation (24%). Other studies have reported that the main cause for rapid $^{15}N\text{-}NH_4^+$ consumption in IPD and tracer immobilization studies were either biotic processes (Bruun et al., 2006; Fitzhugh et al., 2003; Herrmann et al., 2007; Hill et al., 2012; Wilkinson et al., 2014), rapid abiotic fixation (Davidson et al., 1991; Kowalenko and Cameron, 1978), or both biotic immobilization and abiotic fixation (Johnson et al., 2000; Morier et al., 2008; Schimel and Firestone, 1989; Trehan, 1996). To which extent one or the other process prevails depended on factors such as the soil C and N content (Booth et al., 2005), soil moisture (Gouveia and Eudoxie, 2007), soil NH_4^+ fixation capacity, and the clay content and composition (Nieder et al., 2011). We found a higher proportion of the added tracer abiotically fixed in the grassland soil compared to the forest soil. This may have resulted from a higher NH_4^+ fixation capacity in the grassland soil due to higher clay content when compared to the forest soil (clay content: Grassland: 26%; Forest: 16%) in combination with the lower initial NH_4^+ concentration. Davidson et al. (1991) reported similar findings on the importance of abiotic reactions as sinks for $^{15}N\text{-}NH_4^+$ in both forest and grassland soils. Moreover, at higher NH_4^+ concentrations as found in the forest soil compared to the grassland soil (Fig. S1) competition for the cation binding sites in the interlayers of 2:1 clay minerals may be significantly increased, and consequently $^{15}N\text{-}NH_4^+$ being less likely to become bound.

4.2. Reflux from abiotic sinks of NH_4^+

In both soils, abiotic fixation of NH_4^+ was almost exclusively due to the mineral and not the organic fraction of the soil (Table 3). In general, a release of fixed ^{15}N-NH_4^+ by clay minerals could occur in quantities that affect the dynamics of exchangeable NH_4^+ (Matsuoka and Moritsuka, 2011). Both processes, NH_4^+ fixation and the release of NH_4^+ from clay minerals, are mainly controlled by ion diffusion processes (Kowalenko and Cameron, 1978; Nõmmik, 1965; Steffens and Sparks, 1997), and thus primarily depend on the NH_4^+ concentration in the soil solution phase. As the live forest soil showed constant NH_4^+ concentrations over time (Fig. S1) and ^{15}N recovery in the clay fixed soil fraction increased with time (Table 3), we suggest that re-diffusion of fixed ^{15}N-NH_4^+ into the available pool was highly unlikely for this soil. Although in the grassland soil NH_4^+ concentrations decreased over time (which might increase the likelihood of re-diffusion), the constant increase in the ^{15}N recovery rate from the clay fixed N pool over time also points away from a reflux of tracer from clay interlayers in the grassland soil. Moreover, the release of clay fixed NH_4^+ into soil solution is a slow process, previously being suggested to take weeks to years (Kowalenko and Cameron, 1978; Nieder et al., 2011). Therefore, there is no need to consider and evaluate the reflux of clay fixed ^{15}N-NH_4^+ in short-term IPD experiments lasting up to two days. Furthermore, it is unknown whether clay fixation of ^{15}N-NH_4^+ is concomitant with the reciprocal release of native, unlabeled fixed NH_4^+, which would result in an overestimation of gross N mineralization rates.

We found a small fraction of the abiotically fixed ^{15}N-NH_4^+ bound to the humic fraction of the soils (1.1% after 24 h in the grassland soil, Table 3). This might be due to the covalent bonding of NH_4^+ in the form of ammonia (NH_3) to various functional groups in humic substances, such as ketones, or alternatively due to physical condensation reactions of phenolic hydroxyls, hydroquinones and quinone polymers with NH_3 (Burge and Broadbent, 1961; Nõmmik and Vahtras, 1982; Stevenson, 1994; Thorn and Mikita, 1992). Covalent bonding of ammonia to soil organic matter is expected to result in fairly stable compounds that are only slowly mineralized by soil microorganisms (Monaghan and Barraclough, 1995; Thorn and Mikita, 1992). Since bonding to humic substances was minimal in this study, and degradation is supposed to be slow, we deduce that there is no need to consider the re-mineralization of humic fixed ^{15}N-NH_4^+ as a source for reflux in this study. Our findings on the contribution of the mineral and the organic fraction to NH_4^+ fixation are also consistent with the results of the few other studies available (Kowalenko and Cameron, 1978; Nõmmik and Vahtras, 1982; Trehan, 1996).

Interestingly, in the forest soil, the recovery rate of ^{15}N in the DON pool increased significantly over time (Fig. 2). The formation of DON, a heterogeneous mixture of compounds (Farrell et al., 2011), results from a complex mix of biotic and abiotic processes (Neff et al., 2003). Biotic formation of ^{15}N labeled DON can result from microbial NH_4^+ assimilation and exudation or cell lysis (Seely and Lajtha, 1997), which in our experiment, due to the low amount of ^{15}N-NH_4^+ taken up by microbes in the forest soil (Fig. 2), seems to contribute only to a minor extent. However, abiotic fixation by the low-molecular weight organic fraction of the soil, similar to bonding with humic substances as described above, could also explain the observed increased ^{15}N tracer recovery in DON. In the case of the forest soil, an argument against the covalent bonding of the labeled NH_4^+ would be the low soil pH of 4. Covalent bonding of NH_4^+ to organic compounds has only been reported in the form of NH_3, which only becomes the dominant form relative to NH_4^+ in soils under alkaline conditions (Burge and Broadbent, 1961; Thorn and Mikita, 1992). Moreover, only few studies reported on the biodegradability of DON and on DON mineralization (Jones et al., 2004). Jones et al. (2004) suggested that the low-molecular weight fraction of DON comprises only 10–30% of all DON but may regulate the rate of N mineralization and nitrification in soil directly, serving as a microbial substrate (Jones et al., 2004; Wilkinson et al., 2014). DON often represents 30% or more of the total dissolved N in soil solution or soil extracts (Christou et al., 2005; Farrell et al., 2011) and low-molecular weight organic compounds can be taken up by the microbial community within minutes (Hill et al., 2012; Wanek et al., 2010; Wilkinson et al., 2014). Future studies should therefore investigate DON mineralization when considering possible refluxes of tracer (e.g. from the DON pool) into the available NH_4^+ pool, especially in IPD experiments lasting longer than one or two days.

4.3. Reflux from biotic sinks of NH_4^+

We found that only the recovery rate of ^{15}N from the microbial N pool decreased (by 40–75%) relative to the initial time point over a 24-h incubation period (Fig. 2). Our sensitivity analysis revealed that any reflux of ^{15}N-NH_4^+ taken up by microbes into the soil NH_4^+ pool likely had a negligible impact in the forest soil during this period (Table 4). Specifically, our simulations suggested that the gross N mineralization rate of the forest soil could be underestimated by a maximum of 3%. In contrast, simulations suggested that the gross N mineralization rate of the grassland soil could be underestimated in the worst-case scenario by a maximum of 63% (Table 4), assuming all of the rapidly consumed microbial ^{15}N would reflux into the soil pool as $^{15}NH_4^+$. The low impact of simulated microbial N reflux in the forest soil is explained by the low amount of ^{15}N-NH_4^+ taken up by microbes relative to the large NH_4^+ pool. In the grassland soil, a reflux of the high amount of ^{15}N-NH_4^+ taken up by microbes, combined with the low NH_4^+ concentration in this soil, had a large impact on the estimation of gross NH_4^+ transformation rates. Despite this, such a large reflux of ^{15}N taken up by microbes in the grassland soil seems unlikely during an experimental period of only 24 h. Fast efflux of unmetabolized ^{15}N-NH_4^+ from cells is always coupled to cellular influx (uptake) of NH_4^+ (Ludewig et al., 2007; Morgan and Jackson, 1988), and channel- or carrier-mediated NH_4^+ efflux from microbial and plant cells has been reported (Hadas et al., 1992; von Wirén and Merrick, 2004). The fraction of NH_4^+ taken up and subsequently lost by efflux is negatively related to NH_4^+ assimilation, and decreases at low substrate concentrations in plants (Forde and Clarkson, 1999). We therefore suggest that the amount of $^{15}NH_4^+$ efflux is minimal under the N limited conditions of the grassland soils, due to its low NH_4^+ concentration (see also Bengtson and Bengtsson, 2005), fostering microbial assimilation rather than efflux of NH_4^+.

In contrast, re-mineralization of microbial N, that was previously taken up and assimilated into organic N, would be a much slower process than microbial NH_4^+ efflux. However, re-mineralization of organic ^{15}N originating from microbes could potentially represent a significant source of tracer reflux in the grassland soil, at rates impacting the gross N mineralization rate. The rapid incorporation and assimilation of ^{15}N-NH_4^+ into microbial biomass could explain the decrease in ^{15}N enrichment and ^{15}N recovery in the microbial N pool over time (Fig. 2, Fig. S2), while the microbial biomass N content, at least for the grassland soil, increased significantly over incubation time (Fig. S1). This may be explained by continued microbial NH_4^+ uptake with decreasing ^{15}N enrichment over time (Fig. S2) or by technical constraints arising from the sCFE method. It is impossible to extract the total amount of ^{15}N taken up by microbes with the sCFE extraction as applied in this experiment, especially if ^{15}N-NH_4^+ was metabolized and built into insoluble cellular components such as cell walls (Fierer and Schimel, 2003). In general, the application of chloroform extraction methods only enables the measurement of soluble N compounds within microbial cells and not insoluble compounds such as cell wall proteins or peptidoglycans (Jenkinson et al., 2004). This means that in grassland soils we would be facing continuous uptake of NH_4^+ from soil solution, in combination with ongoing removal from the extractable N_{mic} pool as microbes produce insoluble cell components. This would ultimately result in the decrease in the ^{15}N recovery rate from the N_{mic} pool, as found in both soils, rather than indicating ^{15}N reflux or re-

mineralization from the microbial N pool. Usually, microbial NH_4^+ uptake and assimilation, turnover (lysis) and re-mineralization of microbial N is assumed to take from a few days to weeks (Herman et al., 2006; McGill et al., 1975), which means that in a short-term laboratory incubation of 24 h as applied in our IPD experiment, re-mineralization of assimilated ^{15}N-NH_4^+ is relatively slow. Microbial turnover rates in temperate forest and grassland soils have been found to range between 0.004 and 0.03 d^{-1} (corresponding to microbial turnover times of 30–220 days; Spohn et al., 2016a, 2016b), also playing against a strong reflux of tracer from the microbial ^{15}N pool due to slow turnover of microbial biomass.

Therefore, our sensitivity analysis indicates that the reflux of recently taken up ^{15}N tracer from the microbial N pool could potentially have a large impact on the estimation of gross N mineralization rates, but significant reflux appears to be unlikely during incubation periods of about 24 h. These findings are in line with other studies, for example Bengtson and Bengtsson (2005), who showed that in IPD experiments, re-mineralization is lowest during the first two days of incubation. Also others (Barraclough, 1995; Bjarnason, 1988; Davidson et al., 1991; Herrmann et al., 2007; Murphy et al., 2003; Wang et al., 2001) found that re-mineralization is negligible in IPD experiments during incubation times between 24 h and up to a few days, even in studies on rapidly immobilizing grassland soils (Davidson et al., 1991).

Nonetheless, given the potential impact of reflux on gross N transformation rates, re-mineralization fluxes should be measured directly and accounted for in the IPD calculations. Though re-mineralization is hard to quantify directly in soil (but has been done in soil microbial cultures; Bengtson and Bengtsson, 2005) we here propose three approaches to assess its magnitude quantitatively in soils: (1) Gross N mineralization fluxes apparently decline over time due to increasing reflux of microbial ^{15}N from biomass turnover, given the time lag of this reflux relative to microbial NH_4^+ immobilization. The decrease in gross N mineralization fluxes over time can be solved analytically for different time intervals e.g. 4–12 h, 12–24 h, 24–48 h and 48–96 h and then be extrapolated to the study period of 4–24 h. Alternatively this increasing reflux effect can be solved by numerical modeling approaches such as by the Ntrace model (Rütting et al., 2011), the FLUAZ model or others (Bengtson and Bengtsson, 2005; Bjarnason, 1988). (2) Bjarnason (1988) and Herrmann et al. (2007) applied $^{15}NO_3^-$ separately in N mineralization experiments to follow its immobilization, assimilation and the production of $^{15}NH_4^+$ as an index of microbial N re-mineralization. However, this approach targets only the part of the microbial community that actively assimilates NO_3^- and cannot distinguish between re-mineralization of microbial N and dissimilatory nitrate reduction to ammonium (DNRA) that produces NH_4^+ from NO_3^- as major energy conserving mechanism. Parallel measurements of $^{15}N_{mic}$ might resolve some of these issues as DNRA organisms putatively represent only a small fraction of the heterotrophic microbial community and therefore contribute little to $^{15}NO_3^-$ immobilization. Moreover, this approach provides only net rates and numerical or analytical solutions need to be used to derive gross rates of re-mineralization. (3) A third option has recently become amenable, based on direct measurements of rates of soil microbial gross growth and microbial biomass turnover and was applied to soil microbial C dynamics (Spohn et al., 2016a, 2016b). This latter approach could be applied to gross N mineralization experiments and is based on quantifying the ^{18}O incorporation from added ^{18}O-H_2O into double stranded DNA (which is only produced during microbial growth) and conversion of microbial DNA production estimates to N_{mic} and microbial N allocation to growth by CFE. These data would allow the calculation of microbial mortality rates at constant microbial biomass and gross rates of N release from N_{mic}. The third approach has however so far not been applied to such settings, and instead of quantifying the re-mineralization bias in gross N mineralization studies allows the partitioning between gross N mineralization from organic N in microbial biomass/necromass ("re-mineralization") from that of organic N stored in more stable humic substances.

4.4. Consistency of NH_4^+ transformation rates over time

Since constant process rates are a prerequisite for estimating gross N transformation rates (Kirkham and Bartholomew, 1954), we investigated the consistency of transformation rates over time. For the forest soil, transformation rates were approximately constant from 3.5 h after tracer addition until up to 48 h of incubation. In the grassland soil, process rates were approximately constant from 15 min to 24 h of incubation. Initial transformation rates (Fig. 3) were much faster prior to these periods, showing that gross N mineralization rates were systematically overestimated during the shortest incubation period. This is likely due to the lack of equilibration of the added ^{15}N-NH_4^+ (tracer) with the native ^{14}N-NH_4^+ pool (tracee) (Bjarnason, 1988; Watson et al., 2000), and relates to another key assumption of the IPD approach, namely that tracer and tracee behave in the same way in soils (Kirkham and Bartholomew, 1954). Preliminary studies of the time kinetics of consumptive processes and of the tracer/tracee mixing are thus of great importance to find a balance between: (i) the initial time needed to achieve tracer mixing with the native pool (and thereby achieving an identical behavior of the tracer and the tracee); and (ii) the extent of depletion of the ^{15}N pool by consumptive processes. In the grassland soil, ^{15}N-NH_4^+ was almost fully depleted after only 24 h, which is also often observed in other soils (Booth et al., 2005). Such a time frame does not allow for an equilibration time of 24 h before initial sampling as recommended by many authors (Cliff et al., 2002; Herrmann et al., 2007; Murphy et al., 2003; Watson et al., 2000). In the case of rapid depletion of the ^{15}N pool, the use of nitrification inhibitors such as acetylene has been suggested by some authors in order to slow down NH_4^+ immobilization and to prolong incubation time (Herrmann et al., 2007; Murphy et al., 2003). This has proven to be a viable solution for soils showing high nitrification potential (Herrmann et al., 2007), but does not prevent the continuous NH_4^+ fixation occurring due to clay minerals as found in our soils. Also, some non–linear models, developed to calculate gross rates for inorganic N pools that turn over within a day, assume nitrification to be the only consumptive process for ammonium (Davidson et al., 1991), which is not in line with our findings. In our study, a uniform mixing of the tracer solution with the soil NH_4^+ and the equilibrium of tracer and tracee seemed to be reached after an incubation time of only a few h (Barraclough, 1995; Di et al., 2000). Therefore, the estimation of gross N mineralization rates seemed to be justifiable for a time interval between 3.5 h and 24 h in both soils and should, at least in the grassland soil, not exceed 24 h, since errors become more significant as ^{15}N enrichments close to natural abundance levels are approached (Davidson et al., 1991).

5. Conclusion

Overall, we found that biotic immobilization and clay fixation are responsible for the fast consumption of ^{15}N-NH_4^+ in both studied soils while humic fixation played a negligible role. Most importantly, we showed that reflux of rapidly consumed ^{15}N-NH_4^+ was relatively unlikely during our short-term laboratory IPD assay. But one should keep in mind, as Wang et al. (2001) also state, that re-mineralization is part of the continuous process of N mineralization–immobilization and N turnover, both of which determine the net release and availability of inorganic N in soil (Murphy et al., 2003; Wang et al., 2001). Thus, depending on the primary objective of the study, one must choose the appropriate experimental design and duration, and also the appropriate approach for estimating gross N mineralization, either an analytical solution sensu Kirkham and Bartholomew (1954), or a combination of ^{15}N tracing studies coupled to analyses *via* process–based models (Andresen et al., 2015; Cliff et al., 2002; Rütting et al., 2011; Tietema and Wessel, 1992). However, knowing about the inherent assumptions and potential problems of the IPD approach, taking care in applying the

method in the right way and testing the system before applying the IPD assays, allows to estimate gross N mineralization rates (and other soil N processes) in a reliable way.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2017.11.005.

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