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**“Getting to the bottom of ^{15}N Isotope Pool Dilution
technique - Revisiting Gross N Mineralization”**

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Contents

Acknowledgements	1
I. General Introduction	3
1. The internal and external N cycle	5
2. Human impact on the N cycle	7
3. Determination of gross N transformation rates	11
4. Limitations to the isotope pool dilution method	13
5. Applications of the isotope pool dilution method	17
Bibliography	19
II. Manuscript	27
Abstract	29
1. Introduction	31
2. Material and methods	35
2.1. Sampling site and soil description	35
2.2. Experimental design	37

Contents

2.3. Soil sample preparation and sterilization	39
2.4. Tracer addition – start of incubation	40
2.5. Extraction and sCFE – incubation stop	41
2.6. Sample analysis and calculations	42
2.7. Statistical analyses	49
3. Results	51
3.1. Enrichment in ^{15}N and recovery of the tracer from different N pools	51
3.2. Assessment of ^{15}N reflux sources and the importance of eventual reflux	56
3.3. Linearity of NH_4^+ transformation rates	58
3.4. Gross transformation rates of NH_4^+	60
4. Discussion	61
4.1. Recovery of the $^{15}\text{N}\text{--}\text{NH}_4^+$ tracer	61
4.2. Reflux evaluation of the abiotic sinks for NH_4^+	63
4.3. Reflux evaluation of the biotic sinks for NH_4^+	66
4.4. Linearity of NH_4^+ transformation rates	68
4.5. Gross transformation rates of NH_4^+	69
5. Conclusion	71
Bibliography	75
III. Appendix	87
1. Supplementary material	89
2. Zusammenfassung	95

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Part I.

General Introduction

1. The internal and external N cycle

Nitrogen (N) is among the six elements (H, C, N, O, S, and P) from which all major building blocks for life such as DNA, RNA, and proteins are composed (Schlesinger, 1997). The Earth's largest reservoir for N is the atmosphere, consisting of about 78 % of N_2 , which is a highly inert gas (Ward, 2012). The only biological process that makes N_2 accessible for the synthesis of biological macromolecules in terrestrial ecosystems is nitrogen fixation. During this fixation process, N_2 is reduced to ammonium (NH_4^+) and subsequently available for all other organisms (Falkowski et al., 2008). Biological N fixation is carried out by a wide variety of bacteria and archaea and by symbioses such as the well-known bacterial endosymbionts in legumes. Beside biological N fixation, lightning, emissions from volcanoes, and atmospheric wet deposition, are the only natural sources of reactive N in terrestrial ecosystems (Vitousek et al., 2013).

The oxidation and reduction of fixed N fuels the so-called "internal N cycle" (Hart et al., 1994), which is mainly driven by the domain of microbes. This internal N cycle is composed of various transformation processes of N between its stable chemical forms and its various N pools within an ecosystem (Jansson, 1958; Hart et al., 1994).

The vast majority of microbes assimilates ammonium into biomass via amino acids and nucleotides during autotrophic and heterotrophic growth (Ward, 2012). Microbial assimilation and nitrification are the two consumptive processes which immobilize NH_4^+ biotically from the soil. Nitrification refers to the oxidation of

1. The internal and external N cycle

NH_4^+ in a two-step process via nitrite to nitrate for energy generation. Certain autotrophic archaea and bacteria, so-called nitrifiers, utilize the reducing power of NH_4^+ to assimilate carbon dioxide (CO_2). Nitrogen also plays a major role in plant nutrition and regulates primary production. Thus, in terrestrial ecosystems, microbes, as well as plants, incorporate inorganic N into organic matter, which is returned to the ecosystem by the decomposition of dead biomass. These detrital inputs from microbes and plants are to a certain degree taken up by plants and microbes in the form of dissolved organic nitrogen (DON) or are enzymatically converted into inorganic N (Neff et al., 2003; Schimel and Bennett, 2004). Thereby, both intracellular- and extracellular enzymatic deamination of organic N forms release NH_4^+ into the environment. The transformation process of organic N to inorganic N, which makes NH_4^+ available for subsequent assimilation or other consumptive processes, is called N mineralization (Ward, 2012).

In contrast to biological immobilization, other processes removing ammonium from the soil solution are considered abiotic reactions. Abiotic fixation is mainly referred to fixation by clay minerals, in particular by montmorillonites, illites and vermiculites. Hereby, NH_4^+ diffuses into the 2:1 clay mineral interlayers, which subsequently collapse and trap NH_4^+ for periods of weeks to years (Nõmmik and Vahtras, 1982; Nieder et al., 2011; Cavalli et al., 2015). Also, physical condensation reactions of ammonia (NH_3) with phenols or chemical sorption to organic matter remove NH_4^+ from the soil solution (Nõmmik, 1965; Burge and Broadbent, 1961; Thorn and Mikita, 1992).

As soon as N is lost from the ecosystem, it re-enters the external N cycle. Thus, processes removing N finally from an ecosystem are, for example, the leaching of nitrate and its subsequent runoff or microbial denitrification, the conversion of nitrate to the gaseous forms NO and N_2O and, ultimately, N_2 . Nonetheless, also direct gaseous losses of ammonium in the form of ammonia can contribute to the removal of N.

2. Human impact on the N cycle

The natural N cycle consisting of the internal and the external N cycle, has changed drastically over the last decades (Galloway et al., 2004). For some 2.5 billion years, ever since the modern nitrogen cycle evolved, human activities have had the largest impact on the N cycle (Canfield et al., 2010) and have altered it more than any other biogeochemical cycle of importance (Ward, 2012). Humans have changed the Earth system to such an extent that recent and currently forming geological deposits include a signature that is distinct from those of the Holocene and earlier epochs, and will remain in the geological record (Waters et al., 2016). Such stratigraphic signals are the formal prerequisite for the formalization of a new epoch. For this new era shaped by humans, the term “Anthropocene” was introduced by Paul Crutzen in 2002 (Crutzen, 2002). Beside the impact on geological scales, this term also expresses the extent to which humanity is driving rapid and widespread changes to the Earth system that will variously persist and potentially intensify into the future (Waters et al., 2016). The most cited ones of these changes are the rise of greenhouse gases, especially of carbon dioxide (CO₂) and methane (CH₄), and of global temperatures (Waters et al., 2016). Already in 2009, the N cycle was announced as one of the Earth’s processes that has already transgressed its boundary of the “rates of change that cannot continue without significantly eroding the resilience of major components of Earth–system functioning” (Rockström et al., 2009). In the case of the N cycle, the control variable was the rate at which N is fixed by human activity. Vitousek et al. (1997) distinctly outlines the human induced changes on the nitrogen cycle: (i) fertilizer produc-

2. Human impact on the N cycle

tion via the Haber–Bosch process, (ii) biological N fixation in agriculture by the implementation of new agricultural practices, and (iii) the mobilization of fixed N due to fossil fuel combustion, but also due to land–use change (i.e. deforestation and forest fires, drainage of wetlands, conversion of savannas to crop land, etc.). Changes in the N cycle result in increased emissions of N_2O , a greenhouse gas (~ 300 fold more reactive than CO_2) that is increasing steadily in the atmosphere and is currently accounting for about 6 % of all human–caused warming (Galloway and Schlesinger, 2014). Fertilizer use and the promotion of N–fixing legumes in agriculture to satisfy a growing demand for food accounts for the largest contribution to these changes (Canfield et al., 2010). Thus, around 120 million tonnes of N_2 are fixed annually and are introduced into the global N cycle, more than all inputs from Earth’s terrestrial processes combined (Rockström et al., 2009). On a global scale, nearly 90 % of the nitrogen fertilizer is NH_4^+ . Ammonium fertilizer inputs are, to a large extent, oxidized to nitrate. Being more mobile than ammonium in soils, nitrate partly leaches from the soil into aquifers, lakes and rivers, causing eutrophication of coastal waters and creating hypoxic zones in the oceans. During the nitrification process, N_2O is formed as an intermediate, which is emitted from soils and water surfaces, therefore contributing to greenhouse gas emissions (Waters et al., 2016). The majority of these N additions to soils cannot be directly accounted for and are assumed to have accumulated in soils. For example, Waters et al. (2016) estimate that soil nitrogen inventories have doubled in the past century.

More than ever, research on the factors controlling the transformation rates of nitrogen in soil is therefore of fundamental importance for a better understanding of ecosystem behaviour and responses to an anthropogenic–mediated change in the global N cycle (Jones et al., 2004). The N supply to a system and its transformation processes within a system affect the N cycle on an ecosystem level and thus determine the function of many ecosystems (Hart et al., 1994; Vitousek et al., 2002). Ammonium and nitrate is often excessively abundant in agricultural areas and industrial sites, but in many other ecosystems, N is in short supply and de-

notes the other extreme, i.e. N limitation to biological activity (Vitousek et al., 2002). For the development of economical and sustainable nutrient management strategies, N transformation rates in soils need to be taken into account (Jansson, 1958; Di et al., 2000; Eudoxie and Gouveia, 2008). The isotope pool dilution technique provides a powerful tool for the determination of these gross production and gross consumption rates of NH_4^+ and NO_3^- (Hart et al., 1994; Di et al., 2000; Murphy et al., 2003).

3. Determination of gross N transformation rates

The isotope pool dilution method for the estimation of gross N transformation rates is based on the use of the stable heavy isotope ^{15}N . The determination of gross N mineralization rates involves the isotopic labeling of the NH_4^+ (product) pool with $^{15}\text{N}-\text{NH}_4^+$. Respectively, gross nitrification rates are determined via isotopic labeling of the NO_3^- pool. The isotopic tracer can be added to the soil in form of a salt solution like $(^{15}\text{NH}_4)_2\text{SO}_4$ to achieve ^{15}N enrichment of the native NH_4^+ pool. When measuring N mineralization, the ^{15}N enrichment of the labeled product pool is diluted over time by the new production of unlabeled NH_4^+ from organic nitrogen compounds. At the same time, NH_4^+ is removed from the product pool via consumptive soil processes. Since consumption is removing tracer and tracee in equal parts, this process is only changing the NH_4^+ pool size, not the enrichment of ^{15}N . After a certain period of equilibration of the tracer and the native NH_4^+ pool, an initial sampling and a successive final sampling of the soil by salt extraction and filtration is conducted. In each case, NH_4^+ concentration and its ^{15}N enrichment are determined using mass spectrometry. The determination of the change in ^{15}N enrichment as well as the change in NH_4^+ concentrations at both time periods allows one to estimate the gross N mineralization rates in soils. For the estimation of gross nitrification rates, the procedure remains the same; however, nitrogen concentrations and ^{15}N enrichment in NO_3^- are measured in the soil at two time periods.

3. Determination of gross N transformation rates

This set up was developed by Kirkham and Bartholomew (1954), for determining rates of mineralization and immobilization of plant nutrients for the understanding of soil biology itself and also for the “understanding of microbial processes in soil as they are related to plant growth” (Kirkham and Bartholomew, 1954). Thus, the same principle can be applied to phosphate or sulfate transformation rates in soil, using the respective radioisotopic tracers $^{32}\text{PO}_4^{3-}$ and $^{35}\text{SO}_4^{2-}$ (Kirkham and Bartholomew, 1954; Di et al., 2000). Along with the isotope dilution method, Kirkham and Bartholomew (1954) provide the mathematical relationships and equations needed to convert the tracer data, derived from ^{15}N experiments into gross transformation rates. They put three general assumptions forward, namely that: (1) tagged (tracer) and non-tagged atoms (tracee) behave in the same way in soils; (2) mineralization and immobilization rates are constant during the time intervals between successive measurements; (3) immobilized atoms may be neglected as a source of mineralizing material, i.e. re-mineralization is supposed to be so slow and small that it can be neglected.

Jansson (1958) already questioned the latter two assumptions stated by Kirkham and Bartholomew (1954), i.e. that re-mineralization was known to occur to a large extent after prolonged periods of time. This problem prompted Kirkham and Bartholomew (1955) to publish a second set of equations that can be applied where re-mineralization occurs. However, these equations are often not considered since a new assumption of mass conservancy was introduced, which does not apply to many natural systems (Kirkham and Bartholomew, 1955; Bjarnason, 1988; Wang et al., 2001). Therefore, due to the assumptions stated, the equations come along with some limitations. Nevertheless, Kirkham and Bartholomew (1945; 1955) constitute the basis for gross N transformation rate calculations and the same equations have been used ever since, especially during the last 25 years due to the decrease in cost of ^{15}N analysis (Di et al., 2000; Cliff et al., 2002).

4. Limitations to the isotope pool dilution method

The assumptions of the isotope pool dilution method, their relative importance and the implied limitations to the application have been intensely discussed and have been often adjusted to meet the above assumptions (Jansson, 1958; Bjarnason, 1988; Schimel, 1986; Davidson et al., 1991; Barraclough and Puri, 1995; Di et al., 2000; Murphy et al., 2003). Common to all assumptions is the crucial role of the time factor. In the following essay, only a few of the studies on the methodological restrictions that come along with the tracer method will be listed. The first of Kirham and Bartholomew's assumptions, (1) the identical behavior of the tracer and the tracee, is a common assumption to most tracer techniques and can be achieved in isotope pool dilution assays if care is taken with the initial sampling time and a homogeneous labeling of the soil is achieved (Murphy et al., 2003). Here, the time factor applies to the time needed for equilibration of the added tracer with the native pool (tracee). Naturally, isotope pool dilution should cause a non-linear decline in atom % ^{15}N enrichment since the added tracer at first needs to equilibrate with the native pool to attain the same chemical and physical distribution in soil (Bjarnason, 1988; Murphy et al., 2003). In short-term incubation experiments with initial sampling times immediately or only hours after tracer addition, Watson et al. (2000; 2002) and Hermann et al. (2005; 2007) found, for example, that added $^{15}\text{N}\text{-NH}_4^+$ was preferentially immobilized in soil which would result in an overestimation of gross N mineralization rates. They

4. Limitations to the isotope pool dilution method

suggested to conduct the initial sampling 24 hours after ^{15}N addition which was also in agreement with Cliff et al. (2002) and Murphy et al. (2003). Whereas Murphy et al. (1997) found no differences in gross N mineralization estimates when incubation times prior to initial sampling were one, two or four hours. Various soil parameters, such as soil texture and water content, but also the amount and concentration of the added tracer, influence the diffusion of the tracer within a soil (Monaghan and Barraclough, 1995; Cliff et al., 2002; Watson et al., 2000; Booth et al., 2005). Homogeneous isotopic label distribution should thus promote an equilibrium with the native N pool and is therefore required in most experiments (Davidson et al., 1991; Monaghan and Barraclough, 1995; Herrmann et al., 2007).

Non-identical behavior of tracer and tracee also affects time kinetics of the transformation rates and therefore impacts the second assumption of constant rates (2), which have to be considered if a linear model such as Kirkham and Bartholomew's (1954) is applied to calculate gross transformation rates. Nonetheless, the resulting error due to the incorrect description of process rates is probably not large (Bjarnason, 1988; Davidson et al., 1991; Cliff et al., 2002). The marginal error caused is especially small when gross N mineralization rates are low (Recous et al., 1995) and if incubation times are short (Bjarnason, 1988; Watson et al., 2002).

When looking at assumption (3), i.e. no re-mineralization of the ^{15}N tracer, incubation time plays a crucial role. In order to avoid re-mineralization of the tracer, incubation time should not exceed a few days. For example, Bjarnason (1988), Stockdale (1994) suggested not to exceed 6 days of incubation, whereas incubations of 4 days are appropriate according to Schimel (1986). In soil systems with increased temperature (e.g. tropical or subtropical systems), re-mineralization was found to set in earlier (Barraclough and Puri, 1995; Murphy et al., 2003).

The analytical solution provided by Kirkham and Bartholomew (1954) for the estimation of gross N transformation rates therefore have some restrictions. Some of the limitations and weaknesses have been tried to be resolved by the development of improved analyses and numerical models (Mary et al., 1998). Different analyt-

ical solutions vary, for example, in the consumptive processes taken into account when calculating gross N rates. Also non-linear calculations have been developed for special cases, as, for example, when Davidson et al. (1991) developed non-linear equations for the calculation of gross N immobilization rates from ^{15}N dynamics in chloroform-labile microbial biomass. Also, numerical solutions of the differential equations represent a different approach for the estimation of N transformation rates (Myrold and Tiedje, 1986; Wessel and Tietema, 1992; Mary et al., 1998). Numerical models enable the implementation of multiple parameters not accounted for in analytical approaches (e.g. re-mineralization) and can account for various fluxes of ^{15}N simultaneously (Mary et al., 1998; Rütting and Müller, 2007). Furthermore, the assumptions underlying the isotope pool dilution can be examined by numerical models with high precision (Recous et al., 1995; Rütting and Müller, 2007). The combination of new process-based ^{15}N tracing models and actual ^{15}N tracer data can, according to Rütting et al. (2007) provide “more complex and possibly more realistic models and kinetic settings to estimate gross N transformation rates and thus overcomes restrictions of previous ^{15}N tracing techniques”. Moreover, ^{15}N *in situ* tracer studies, combined with numerical data analysis, allows the investigation of gross N transformation processes under field conditions over prolonged periods of time (Rütting et al., 2011; Andresen et al., 2015). However, ^{15}N isotope pool dilution techniques are still the recommended method for data generation underlying all models for gross N transformation processes (Hart et al., 1994; Mary et al., 1998; Rütting et al., 2011).

5. Applications of the isotope pool dilution method

Already in the early stages of isotope tracer studies, Sven Jansson acknowledged isotopic tracers such as ^{15}N as extremely useful for the study of N transformation processes in soils. Conducting ^{15}N tracer experiments, he investigated the soil nitrogen metabolism and, only shortly after the first ^{15}N work on soil nitrogen by Norman and Werkman (1943), Jansson describes the continuous mineralization–immobilization turnover of N and formulated the internal N cycle in soil systems for the first time (Jansson, 1958). To date, the isotope pool dilution has been regularly used to study soil N cycling processes (Wang et al., 2014; Holz et al., 2015) and the effects of environmental factors such as soil N content, soil C–to–N ratio, and microbial biomass content on soil N transformation processes (Bengtson and Bengtsson, 2005; Booth et al., 2005). The influence of individual factors like soil moisture, soil texture, and temperature on N transformation rates in soils have been studied (Murphy et al., 2003) and N cycling in various natural ecosystems has been investigated, broadening the knowledge on the soil N cycle (Accoe et al., 2004; Rütting et al., 2011; Wu et al., 2012; Wild et al., 2015). An increased understanding of the N cycle is helping to improve the predictions of future changes on the N cycle such as increased temperatures (Ma et al., 2011; Andresen et al., 2015). Moreover, considering the growing global demand on food (Canfield et al., 2010), the isotope pool dilution technique is contributing valuable information on the fate of N fertilizer and helps to develop efficient fertilizer N application

5. Applications of the isotope pool dilution method

strategies to increase primary productivity but also in order to avoid N losses to the environment in the form of NO_3^- or N_2O (Watkins and Barraclough, 1996; Di et al., 2000; Sørensen, 2001; Jenkinson, 2001; Chantigny et al., 2004; Shindo and Nishio, 2005; Chantigny et al., 2014; Prommer et al., 2014). Therefore, the isotopic pool dilution technique is, to date, making important contributions on answering these striking environmental research questions.

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Part II.

Manuscript

Abstract

When applying the ^{15}N isotope pool dilution technique for measuring gross N transformation rates, rapid consumption of $^{15}\text{N}\text{--NH}_4^+$ is common in short-term soil incubations. Nevertheless, the processes responsible for rapid consumption are not entirely well understood. The primary objectives of this study is to determine the relative roles of biotic and abiotic processes in NH_4^+ consumption and to investigate the validity of one of the underlying assumption of gross N transformation estimates, i.e. that no reflux of ^{15}N tracer during incubation time occurs. A laboratory experiment was performed, where $^{15}\text{N}\text{--NH}_4^+$ was added to live and autoclaved mineral top soil from a beech forest and a grassland site in Austria, differing in NH_4^+ concentrations and consumption kinetics. The change in concentration and enrichment of the inorganic, the organic, and the microbial N pool was measured at 0, 0.25, 3.5, 24 and 48 hours to investigate biotic consumption processes. Furthermore, abiotic consumption of the isotopic tracer, the contribution of clay fixation and the fixation by humic substances were investigated at 0 and after 24 hours of incubation. We achieved a full recovery of the ^{15}N tracer in both soils over the course of the experiment. In the soil rapidly consuming NH_4^+ , abiotic fixation was the major process removing NH_4^+ only seconds after addition (24 %). But also the uptake by soil microbes (10 %), as well as nitrification (13 %), set in only seconds after tracer addition. Thus being said, we could not detect a violation of the assumption of reflux exclusion, although a sensitivity analysis of ^{15}N tracer reflux revealed a potential impact of such a reflux in laboratory experiments. Our study demonstrated that even in the case of rapid NH_4^+ consumption

during isotope pool dilution experiments, ^{15}N tracer reflux seems to be rather unlikely for short-term incubation of 24 hours, and probably even up to 48 hours, and therefore negligibly affect calculations of gross N mineralization.

1. Introduction

Nitrogen (N), in its inorganic forms ammonium (NH_4^+) and nitrate (NO_3^-), is often described as the limiting nutrient for plants in terrestrial ecosystems (Falkowski et al., 2008). Primary production and many other ecosystem processes are controlled by the rates at which inorganic N is produced via N mineralization and how it is consumed by immobilization and fixation processes. The understanding of this continuous interchange between organic and inorganic nitrogen forms is, therefore, of fundamental importance for estimating the available N in agricultural and natural soil systems (Hadas et al., 1992; Vitousek et al., 2002; Ward, 2012). The isotope pool dilution provides a valuable tool for the determination of these N transformation processes (Barraclough, 1991; Di et al., 2000).

The application of isotope tracer techniques, together with the differential equations developed by Kirkham and Bartholomew in 1954, have enabled soil scientists to estimate both processes i.e. gross mobilization and gross consumption rates of plant nutrients in soil (Kirkham and Bartholomew, 1954). Since then, these equations have been used across a wide range of natural and agricultural systems to study N transformation rates in soil (Hart et al., 1994; Murphy et al., 2003; Booth et al., 2005). To obtain experimental isotope tracer data on gross transformation rates, the ^{15}N isotope pool dilution technique (IPD) is recognized to date as the recommended method (Hart et al., 1994).

In the IPD approach for gross N mineralization, the product pool (NH_4^+) in a soil sample is labeled with $^{15}\text{NH}_4^+$ at an atomic percentage above natural abundance,

1. Introduction

without substantially increasing the NH_4^+ pool size. The successive production of NH_4^+ via mineralization of organic nitrogen at natural ^{15}N abundance dilutes the ^{15}N labeled NH_4^+ pool over incubation time. Gross NH_4^+ production rates and gross NH_4^+ consumption rates can be calculated from the change in size of the total NH_4^+ pool ($^{14}\text{N} + ^{15}\text{N}$), and from the decline in the percentage of ^{15}N atoms above natural abundance over incubation time (Kirkham and Bartholomew, 1954; Barraclough, 1991; Hart et al., 1994; Murphy et al., 2003).

When applying IPD to obtain gross N transformation rates in soil, gross N mineralization (influx) and gross consumption of NH_4^+ (out-flux) is measured (Murphy et al., 2003). Thus, all processes removing NH_4^+ from the pool of available NH_4^+ will be referred to forthwith as consumption. Biotic NH_4^+ removal (microbial uptake and nitrification) will be referred to as immobilization and abiotic NH_4^+ removal processes (fixation by the mineral or the organic soil fraction) will be referred to as fixation. Thus, an investigation of the various immobilization and fixation processes usually only becomes important when a violation of the assumptions that occur with the application of the method is suspected.

Kirkham and Bartholomew (1954) state 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) need to behave in the same way in a soil, (ii) mineralization and immobilization rates remain constant during the interval between successive measurements, and (iii) immobilized labeled inorganic N is not re-mineralized during the experimental period.

We assumed that the third of these key assumptions—no recycling of tracer during the experiment—could be violated during IPD experiments if rapid consumption of the tracer takes place. Rapid consumption of $^{15}\text{N}-\text{NH}_4^+$ has been reported to occur in several studies and still it is not completely understood which processes are responsible for the rapid consumption of $^{15}\text{N}-\text{NH}_4^+$ in soils. Specifically, when large fractions (> 50 %) of the tracer disappear within minutes after its addition, it is not

clear which consumption processes are involved and whether recycling of the ^{15}N tracer is possible (Kowalenko and Cameron, 1978; Davidson et al., 1991; Morier et al., 2008). It is well known that such a reflux of ^{15}N tracer into the NH_4^+ pool could lead to an substantial error in calculations, and specifically result in an underestimation of gross rates (Bjarnason, 1988; Davidson et al., 1991; Barraclough and Puri, 1995). Biotic immobilization such as nitrification and assimilation are often assumed to be the dominant consumptive processes in short-term IPD experiments (Monaghan and Barraclough, 1995; Trehan, 1996; Morier et al., 2008). Indeed, microbial uptake of organic compounds within seconds after addition has been reported by several authors (Farrell et al., 2011; Hill et al., 2012; Wilkinson et al., 2014). Nevertheless, others suggested abiotic fixation to be the reason for the consumption of ammonium within minutes after its addition (Davidson et al., 1991; Trehan, 1996; Johnson et al., 2000). In fact, NH_4^+ fixation by clay minerals is well known to happen within hours after ammonium addition (Nõmmik and Vahtras, 1982; Nieder et al., 2011; Cavalli et al., 2015). Also physical sorption or chemisorption to organic matter might be responsible for the removal of $^{15}\text{NH}_4^+$ from the extractable N pool (Mortland and Wolcott, 1965; Nõmmik and Vahtras, 1982; Nieder et al., 2011). In order to assess the potential for re-mobilization of $^{15}\text{N}-\text{NH}_4^+$ in the case of rapid consumption we have to identify the sinks involved.

The objective of this study therefore is to determine the fate of added $^{15}\text{NH}_4^+$ tracer during the usual duration of a short-term ^{15}N -IPD experiment, in two soils that differ in their NH_4^+ consumption rates. Considering all possible sources of tracer reflux, we evaluate whether the third assumption of Kirkham and Bartholomew (1954) is valid during experimental duration and additionally investigate the linearity of transformation rates over time.

We hypothesized that rapidly consumed ^{15}N tracer is mainly subjected to biotic (microbial) immobilization processes (Hill et al., 2012) and, secondly, that the ^{15}N tracer can, therefore, be re-mineralized or released during experimental incubation time.

1. Introduction

In order to differentiate between biotic and abiotic consumption processes, part of the soil samples were sterilized by autoclaving before tracer addition. 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 forty-eight hours. In addition, the contribution of abiotic fixation was determined at two fixed time points. This resulted in a high time resolution of the change in N pool sizes and their ^{15}N enrichment and tracer recovery over time, allowing us to track the fate of the tracer as well as to calculate gross N transformation rates for various time intervals.

2. Material and methods

2.1. Sampling site and soil description

Soils were collected from two sites in Austria differing in vegetation and soil pH. Topsoils were sampled from a beech forest (*Fagus sylvatica* L.) in Vienna (N 48.228656, E 16.260713, 382 m.ü.A., Schottenwald) and from an annual grassland in Lower Austria (N 48.049063, E 16.197592, 323 m.ü.A., Mödling). The soils are referred to henceforth as “forest” and “grassland” soil respectively. According to the World Reference Base for Soil Resource (IUSS Working Group WRB, 2015), the forest soil is classified as a dystric cambisol in previous studies (Kaiser et al., 2010) and the grassland soil is categorized as a cambisol (Nestroy et al., 2011).

Samples were taken from the upper 10 cm of 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 CaCl₂ and was 4.0 in the forest and 6.0 in the grassland soil. Total carbon (C) and N contents, as well as C : N ratios and $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values were measured in finely ground, oven dried (105 °C, 24 h) soil using an elemental analyzer (EA 1110, CE Instruments, Milan, IT) coupled to a continuous flow stable isotope ratio mass spectrometer (DeltaPLUS, Thermo Finnigan, Bremen, DE) (EA–IRMS).

For the soil texture analysis, the clay fraction was determined based on a micro-pipette method modified after Miller and Miller (1987). For this purpose, we retrieved 2.5 mL of a soil suspension (4 g air dried soil suspended in 40 mL 5 %

2. Material and methods

sodium hexametaphosphate) from a 50 mL Greiner PP tube (Greiner Bio-One, DE) at the specified depth of 2.5 cm after shaking and two hours of particle settling (Miller and Miller, 1987). The retrieved suspension was dried in a drying oven at 85 °C for 24 h and its dry weight was measured. The sand fraction was sieved out of the residual soil suspension with a 0.063 mm mesh size under running water, dried, and its dry weight was measured. The silt fraction was subsequently estimated by the subtraction of the clay and sand fraction from the overall soil dry weight. The soil water content (SWC) was determined gravimetrically (drying at 105 °C for 24 h).

Table 2.1.: Soil characteristics in the top soil (0-10 cm) of the forest (F) and the grassland soil (G)

	Soil texture			C & N content			
	pH	Clay (%)	Sand (%)	Silt (%)	Total C (%)	Total N (%)	C : N ratio
F	4.0	16.3	20.3	63.4	3.4	0.2	13
G	6.0	26.2	17.7	56.1	2.9	0.3	10

Table 2.2.: NH_4^+ content and recovery rate of added NH_4^+ after 15 min in the forest (F) and the grassland soil (G)

	NH_4^+ concentration	^{15}N tracer recovery
	($\mu\text{g N g}^{-1}$ soil dry weight)	(after 15 min) (%)
F	16.0	99
G	4.1	41

Shortly before the main isotope pool dilution experiment, the soil NH_4^+ content was determined colorimetrically in soil extracts (4 g fresh weight : 30 mL 1M KCl), as based on the Berthelot reaction (Hood-Nowotny et al., 2010). In a tracer recovery experiment, the fresh forest and grassland soils were labeled with 10 atom % $^{15}\text{N}-\text{NH}_4^+$ and after 15 min extracted with 0.5 M K_2SO_4 (4 g fresh weight : 30 mL extractant). Ammonium was collected by microdiffusion with MgO in acid traps

(see 2.6.1) and measured via EA–IRMS.

Both soils were similar in the total C, N and water content. According to Miller and Miller (1987), particle size distribution classify both soils as silt loam soils (Table 2.1). The total C and N content in the forest soil was 3.4 % and 0.2 % respectively, with a C to N ratio of 13. The grassland soil had a total C content of 2.9 % and a total N content of 0.3 %, with a C to N ratio of 10. Both soils had similar SWC values ($0.27 \text{ g H}_2\text{O g}^{-1}$ soil dry weight for the forest and $0.21 \text{ g H}_2\text{O g}^{-1}$ soil dry weight for the grassland soil). Water holding capacity (WHC), calculated from water saturated soil and soil dry weight, was estimated at $0.7 \text{ g H}_2\text{O g}^{-1}$ soil dry weight for the forest soil and $0.78 \text{ g H}_2\text{O g}^{-1}$ soil dry weight for the grassland soil. Selected values are given in Table 2.1. The forest soil showed much higher NH_4^+ concentrations ($16.0 \mu\text{g N g}^{-1}$ soil dry weight) than the grassland soil ($4.1 \mu\text{g N g}^{-1}$ soil dry weight). The tracer recovery experiment showed that after 15 min of incubation, 99 % of the added ^{15}N tracer could be recovered from the forest soil but only 41 % from the grassland soil (Table 2.2). The two soils were therefore chosen because of their similarities in general soil properties, such as texture and C and N content. However, both soils differed greatly in soil pH, the available NH_4^+ content, and in their consumption of $^{15}\text{N}-\text{NH}_4^+$.

2.2. Experimental design

The main IPD experiment was conducted on 4 g aliquots of the bulk soil samples of the forest and the grassland. The IPD assay was performed with both sterilized and living (non–sterilized) soil. We treated the fresh soils with two autoclaving cycles in order to distinguish between abiotic and biotic immobilization and transformation processes. All samples were isotopically labeled with a 10 atom % $^{15}\text{N}-\text{NH}_4^+$ tracer solution. The added concentration increased the total ammonium pool by a maximum of 20 %. A second set of samples was treated similarly, but when it came to extracting the soils, liquid chloroform (CHCl_3) was added along with the

2. Material and methods

extractant. This was added to perform a simultaneous chloroform–fumigation extraction (sCFE) at the end of the incubation period. The sCFE was applied in order to estimate the amount of ^{15}N assimilated by microbes during the experiment. To obtain high–resolution time kinetics of the ^{15}N immobilization, fixation and transformation processes, incubation times of the IPD experiment were measured in five stages. Incubations were stopped immediately after label addition (0 h), as well as after 15 min (0.25 h), 3.5 h, 24 h, and 48 h after label addition. We thereby used the standard experimental duration as suggested by Murphy et al. (2003) at which the first sampling point is normally set between 4 h and 24 h depending on the soil properties, whereas the second sampling point is suggested to be after a maximum of two to six days (Bjarnason, 1988; Davidson et al., 1991; Di et al., 2000; Murphy et al., 2003). We included the first two sampling points to also track rapid consumptive processes. A schematic diagram of the experimental set–up can be seen in Figure 2.1.

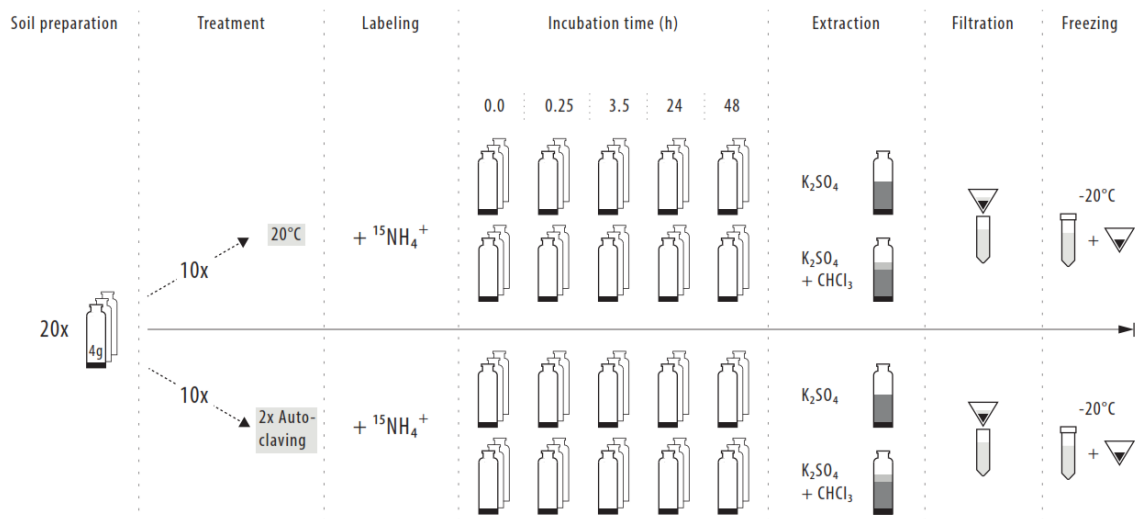


Figure 2.1.: Experimental set-up

2.3. Soil sample preparation and sterilization

In order to condition the soils for the IPD experiment, the soil water content was adjusted to approximately 50 % WHC. This was done to minimize the effect of the change in soil moisture on the activity of soil microorganisms, which is greatest when label is added to dry soil (Di et al., 2000). Therefore, prewetting is desirable to avoid a short-term flush of N mineralization during incubation time of the experiment (Davidson et al., 1991; Stark and Firestone, 1995; Cliff et al., 2002; Fierer and Schimel, 2003). Furthermore, a higher soil water content was shown to increase the sterilization effectiveness (Wolf and Skipper, 1994; Trevors, 1996). After adjustment of SWC to 50 % WHC (~ 1 h), 4 g of soil was weighed into 50 mL glass vials (Crimp Top Headspace Vials, Supelco, US) and covered with parafilm. The soils were prepared in triplicates for each time period, and also for the treatment and extraction methods. Sterilization of part of the soil samples was necessary in order to separate the biotic from abiotic consumption processes. The soil samples set aside for sterilization were covered with aluminum foil and autoclaved at 121 °C for 20 min after 24 hours. To ensure the elimination of biotic activity, soil samples were autoclaved twice, as suggested by Wolf et al. (1989). The first heating cycle was conducted 48 h before tracer addition and the second cycle 2 h before tracer addition. The samples were allowed to cool down to room temperature after each heating cycle and the water content was checked gravimetrically. We chose autoclaving because our previous experiments showed that it is more efficient than amending the samples with HgCl_2 and NaN_3 or treating them with CHCl_3 (data not shown). Autoclaving, like most sterilizing procedures, changes the chemical and physical properties of soil. For example, NH_4^+ and NO_3^- concentrations increase significantly in autoclaved soil (Lopes and Wollum, 1976; Wolf and Skipper, 1994). Nevertheless, it has a negligible impact on the cation exchange capacity or the soil pH; moreover, the handling is safe, and autoclaving is easily applicable (Wolf et al., 1989). All flasks, pipette tips, and rubber septa were

autoclaved prior to the experiment (20 min, at 121 °C), to keep samples as sterile as possible throughout the incubation period. In order to avoid contamination of the sterilized samples by ambient air, all tracer injections were conducted in close proximity to a Bunsen burner within the "sterile field" (Skipper et al., 1996; Sanders, 2012).

The soils not exposed to sterilization were kept at 20 °C during the same pre-treatment period.

2.4. Tracer addition – start of incubation

In order to track the fate of NH_4^+ in the soils, each sample was labeled with a 10 atom % $(^{15}\text{NH}_4)_2\text{SO}_4$ solution differing in NH_4^+ concentration. Each concentration depended on the soil NH_4^+ concentrations previously measured in the soils and further validated in the pretest. The aim was, on the one hand, to increase the product pool as little as possible (thus avoiding stimulation of microbial NH_4^+ immobilization processes) and, on the other hand, to enrich the NH_4^+ pool sufficiently with $^{15}\text{NH}_4^+$ to determine with high precision the rate of isotope dilution (Davidson et al., 1991; Di et al., 2000). The two label solutions were prepared with 10 atom % $(^{15}\text{NH}_4)_2\text{SO}_4$ (Sigma Aldrich) and with deionized ultrapure water ($>18.2 \text{ M}\Omega \text{ cm}^{-1}$, MilliQ, Millipore). We added a 0.5 mM 10 atom % $(^{15}\text{NH}_4)_2\text{SO}_4$ solution to the forest soil and a 0.1 mM 10 atom % $(^{15}\text{NH}_4)_2\text{SO}_4$ solution to the grassland soil, increasing the original soil NH_4^+ pool by a maximum of 20 %. The solutions were applied across the soil surface in multiple drops using a pipette. Both soils received a total volume of $400 \mu\text{L}$ of tracer solution with the intention to ensure homogeneous labeling of the soil, though not exceeding 70 % of the WHC. The vials were covered with parafilm or aluminum foil and carefully shaken by hand, thus facilitating a uniform label distribution throughout the soil and avoiding any systematic bias (Davidson et al., 1991). The application of the ^{15}N solution marked the start of the experiment for all time periods. Soil samples

were incubated at 20 °C in darkness for 0 hours up to 48 hours. Extraction of the soil samples marked the end of the incubation period.

2.5. Extraction and sCFE – incubation stop

The incubation of the soil samples with ^{15}N tracer was stopped by extraction of the soil with 30 mL of a 0.5 M K_2SO_4 solution with and without CHCl_3 amendment. The vials were capped with air tight butyl septa (Supelco, US), fixed with crimp seals (Supelco, US), and shaken horizontally for 30 min at 150 rpm on an orbital shaker. For sCFE, samples were extracted in exactly the same manner, but along with the K_2SO_4 solution, 0.5 mL of EtOH-free CHCl_3 was pipetted onto the soil before closing the vials. The amendment with CHCl_3 resulted in the extraction of soil microbial biomass N (N_{mic}) in addition to the extractable soil N. For the quantification of N_{mic} we chose the sCFE method modified from Gregorich et al. (1990) and Fierer Schimel (2003a), thus avoiding relatively long fumigation periods used in the traditional CFE method (Brookes et al., 1985; Tate et al., 1988). This could, according to Fierer (2003b), lead to a significant disagreement in N levels between non-fumigated and fumigated samples, especially in short-term ^{15}N labeling experiments, on account of an incomplete inhibition of biological activity in soils during the CFE process. Nevertheless, instantly after tracer addition (0 h), the first set of samples was extracted (with and without CHCl_3). Following extraction, all soil suspensions were gravity filtered through Whatman filter papers (ashless, Grade 40, WhatmanTM, GE Healthcare, UK).

All filters were pre-rinsed 2–3 times with 0.5 M K_2SO_4 , then rinsed with deionised water, and dried in a drying oven at 60 °C. This was conducted before the experiment to avoid any contamination with NH_4^+ from the cellulose filter paper. N_{mic} was calculated from the difference between total dissolved N (TDN) extracted by 0.5 M K_2SO_4 with and without addition of liquid chloroform, as modified from Brookes et al. (1985). The soil extracts, as well as the extracted soils remaining

in the cellulose filters (see Section 3.2), were each stored at - 20 °C until further analysis was conducted.

2.6. Sample analysis and calculations

2.6.1. Determination of ammonium content, gross mineralization and immobilization rates

All filtered extracts were analyzed for the content and isotopic composition of NH_4^+ , in order to calculate gross mineralization and consumption rates. In preparing the extracts for isotope ratio mass spectrometry, an aliquot of 10 mL was subjected to a microdiffusion in 20 mL PE scintillation vials with screw caps (Sarsted, DE) as described by Lachouani et al. (2010). Briefly, the NH_4^+ in the extract was converted to NH_3 due to an increase in solution pH induced by the MgO amendment (100 mg). Ammonia in gaseous form was diffused into an acid trap, i.e. an acidified filter disk cut out from Whatman filter papers (ashless, Grade 40, Whatman, GE Healthcare, UK; saturated with 2.5 M KHSO_4). This was enclosed in semi-permeable Teflon tape, which was then added to the extract along with MgO. Under the conditions of acid traps, NH_3 dissociates to NH_4^+ and concentrates on the paper disks. The microdiffusions were run on an orbital shaker at 150 rpm for 48 h at room temperature, to allow for the complete diffusion of NH_4^+ into the acid trap (J. Prommer, unpublished data, 2013). After two days, the filter disks were removed from the soil extracts, rinsed with deionized water and dried over concentrated sulfuric acid in a desiccator at 10 mbar vacuum. The dry filter disks were subsequently unwrapped, folded into tin capsules, and subjected to EA-IRMS.

Gross NH_4^+ production (GP) and gross NH_4^+ consumption (GC) were calculated for both treatments (live and sterile), and for all time intervals, according to the equations by Kirkham and Bartholomew (1954),

$$GP = \frac{C_{t1} - C_{t2}}{t_2 - t_1} \times \left(\frac{\ln \left(\frac{APE_{t1}}{APE_{t2}} \right)}{\ln \left(\frac{C_{t2}}{C_{t1}} \right)} \right) \quad (2.1)$$

$$GC = \frac{C_{t2} - C_{t1}}{t_2 - t_1} \times \left(1 + \frac{\ln \left(\frac{APE_{t2}}{APE_{t1}} \right)}{\ln \left(\frac{C_{t2}}{C_{t1}} \right)} \right) \quad (2.2)$$

where t_1 and t_2 represent the time when incubation was stopped (0 h–48 h). C_{t1} and C_{t2} represent the soil NH_4^+ concentrations in $\mu\text{g N g}^{-1}$ soil dry weight at the time the incubation was stopped. Atomic percent excess of ^{15}N (APE) was calculated for t_1 and t_2 as follows:

$$APE = \text{atom } \%^{15}\text{N}_{\text{sample}} - \text{atom } \%^{15}\text{N}_{\text{background}} \quad (2.3)$$

We used 0.3663 atom % for the $^{15}\text{N}_{\text{background}}$ levels, thus corresponding to the natural abundance of ^{15}N in soil. The value for $\text{atom } \%^{15}\text{N}_{\text{sample}}$ was blank corrected based on $\delta^{15}\text{N}$ values, as in the formula below (Equation 2.4, Lachouani et al. 2010). Gross rates were expressed in $\mu\text{g N g}^{-1}$ soil dry weight d^{-1} .

$$\delta^{15}\text{N}_{\text{blank corr}} = \frac{\delta^{15}\text{N}_{\text{sample}} \times \text{area}_{\text{sample}} - \delta^{15}\text{N}_{\text{blank}} \times \text{area}_{\text{blank}}}{\text{area}_{\text{sample}} - \text{area}_{\text{blank}}} \quad (2.4)$$

2.6.2. Estimation of the inorganic, microbial, and organic ^{15}N pools

For the identification of all NH_4^+ sinks, the added ^{15}N in the soil was traced. This was possible by following the change in N content and in isotopic composition over incubation time in the dissolved inorganic N pool (NH_4^+ and NO_3^-), the total dissolved N pool (TDN), and the microbial N pool (N_{mic}). Finally, the concentration and isotopic composition of the dissolved organic N pool (DON) were calculated at all times from the former measurements.

In order to measure the NO_3^- , TDN, N_{mic} , and DON content of the soils, as well

2. Material and methods

as the isotopic composition at all times, we prepared samples for purge-and-trap isotope mass spectrometry (PT-IRMS), consisting of 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). By PT-IRMS, the concentration and isotopic composition of N_2O can be measured, thus the methods applied aim at the specific conversion of the target N compounds to N_2O . For this purpose we made use of the VCl_3 -azide method, converting NO_3^- to N_2O , as described by Lachouani et al. (2010). The conversion of target N compounds to NO_3^- was accomplished by different pretreatments, then with a reduction of NO_3^- by Vanadium (III) chloride (VCl_3) to NO_2^- and with a further reduction to N_2O by the injection of sodium azide (NaN_3) to the sample (McIlvin and Altabet, 2005; Lachouani et al., 2010).

Hence, for the NO_3^- measurements, the samples were subjected only to VCl_3 and NaN_3 treatment, in order to convert extracted NO_3^- to N_2O . Only non-fumigated samples were measured for NO_3^- isotopic composition on the PT-IRMS as reported by Lachouani et al. (2010), with some minor modifications. All $\delta^{15}\text{N}$ calculations were blank corrected using a weighted blank correction, according to the mixing model as described for the NH_4^+ isotopic composition (see Equation 2.4). The correction was performed in a similar way for all subsequent calculations of atom % ^{15}N .

For the estimation of TDN, a pretreatment was required to convert all the dissolved N in the soil extract to NO_3^- , which was then further converted with VCl_3 and azide to N_2O . Alkaline persulfate oxidation was applied to oxidize TDN to NO_3^- , with methods described by Cabrera and Beare (1993) and Doyle et al. (2004). All samples, both fumigated and non-fumigated, were subjected to this procedure. Accomplishing a complete conversion of TDN to NO_3^- was validated by the additional 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).

The PT-IRMS measurements not only enabled us to obtain the isotopic composition of TDN and NO_3^- for all time points, but also allowed us to calculate the N_{mic} content from fumigated (fum) and non-fumigated (non-fum) samples, as proposed by Brookes et al. (1985). This is represented in Equation 2.5, and the isotopic composition of N_{mic} can be calculated using Equation 2.6 (Lachouani et al., 2010).

$$\text{N}_{\text{mic}} = \text{TDN}_{\text{fum}} - \text{TDN}_{\text{non-fum}} \quad (2.5)$$

$$\delta^{15}\text{N}_{\text{N}_{\text{mic}}} = \frac{\text{TDN}_{\text{fum}} \times \delta^{15}\text{N}_{\text{TDN}_{\text{fum}}} - \text{TDN}_{\text{non-fum}} \times \delta^{15}\text{N}_{\text{TDN}_{\text{non-fum}}}}{\text{TDN}_{\text{fum}} - \text{TDN}_{\text{non-fum}}} \quad (2.6)$$

However, 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). Besides, K_{EN} factors for these soils under these incubation conditions were unknown and did not allow us to apply a conversion factor to account for the non-labile assimilated ^{15}N (Davidson et al., 1991).

Combining the results from EA-IRMS and PT-IRM measurements, we estimated the DON content using Equation 2.7, and its isotopic composition using Equation 2.8. We followed the assumption that subtracting inorganic N content from TDN yields the extractable organic fraction of soil N:

$$\text{DON} = \text{TDN} - (\text{NO}_3^- + \text{NH}_4^+) \quad (2.7)$$

$$\delta^{15}\text{N}_{\text{DON}} = \frac{\text{TDN}_{\text{non-fum}} \times \delta^{15}\text{N}_{\text{TDN}_{\text{non-fum}}} - (\text{NO}_3^- \times \delta^{15}\text{N}_{\text{NO}_3^-} + \text{NH}_4^+ \times \delta^{15}\text{N}_{\text{NH}_4^+})}{\text{DON}} \quad (2.8)$$

Contents of soil NO_3^- , TDN, N_{mic} , and DON were calculated on a dry weight basis in $\mu\text{g N g}^{-1}$ soil dry weight. All isotope ratios are given in atom % ^{15}N .

2.6.3. Calculation of enrichment in ^{15}N and recovery of ^{15}N

Beside the N content, we also determined the atom % ^{15}N enrichment for all quantified N fractions (NH_4^+ , NO_3^- , N_{mic} , DON) over incubation time, thus following the increase or decrease of enrichment in ^{15}N . At first, $\delta^{15}\text{N}$ values were calculated using Equation 2.9.

$$\delta^{15}\text{N} = \left(\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000 \quad (2.9)$$

R represents the ^{15}N to ^{14}N ratio in a sample and a standard and the δ values are expressed relative to the reference of atmospheric air, in per mill,

These $\delta^{15}\text{N}$ values were used for blank corrections, followed by the conversion of the blank corrected δ values to atom % ^{15}N , as seen in Equation 2.10

$$\text{atom \%} = \frac{100 \times \text{AR} \times \left(\frac{\delta}{1000+1} \right)}{1 + \text{AR} \times \left(\frac{\delta}{1000+1} \right)} \quad (2.10)$$

where AR represents the absolute ratio (0.003663) for the atmospheric air- N_2 reference.

Furthermore, in order to investigate the fate of the ^{15}N tracer, we estimated the recovery rate of the ^{15}N ($^{15}\text{N}_{\text{recovered}}$) added to the soil as the total amount of ^{15}N recovered divided by the amount injected, expressed in % as suggested by Hart

et al. (1994):

$$^{15}\text{N}_{\text{recovered}} = \frac{^{15}\text{N excess } (\mu\text{g g}^{-1} \text{ soil dry weight}) \times \text{sample dry weight (g)}}{^{15}\text{N added } (\mu\text{g})} \times 100 \% \quad (2.11)$$

In this formula, ^{15}N excess values represent the APE as calculated in Equation 2.3 and is relative to the N pool size.

2.6.4. Determination of inorganic and organic fixed nitrogen pools

All estimations described in Section 2.6.1 and 2.6.2 are based on soil extract analyses to determine inorganic and organic nitrogen pools and are mainly controlled by biotic processes. The results enable us to assess the N content and isotopic composition of extractable N and of N immobilized by soil microbes at all incubation times in both live and sterile soils. Since we were not able to completely recover the added ^{15}N from the pools, we also investigated non-extractable N in the solid phase of the soil samples by the following method.

Given that the fumigated soil extracted with K_2SO_4 solution would solely hold abiotically fixed NH_4^+ , we determined the total fixed N (TN_{fixed}) content and isotopic composition in the forest and the grassland soils in samples subjected to fumigation during the experiment. We investigated the samples extracted immediately after tracer addition (0 h), and after 24 h of incubation for the live soils and after 24 h of incubation for the sterile soils. After thawing and homogenizing the frozen and extracted soil residues cautiously with a spatula, aliquots of 100 mg were weighed into 2 mL Eppendorf tubes (safe lock tubes, Eppendorf AG, DE). In order to eliminate the remaining extractant and extractable N, a washing step with 1.5 mL of ultrapure water was carried out. Subsequently, samples were shaken on an orbital shaker for 15 min at about 150 rpm and centrifuged at $1500 \times g$ for 10 min. Afterwards, the supernatant was discarded and the tubes were left to dry at 60°C for two days, then ground to a fine powder in a ball mill.

2. Material and methods

Aliquots of the ground soil samples were weighed into tin capsules and subjected to EA–IRMS, in order to analyse the nitrogen isotopes in TN_{fixed} . Control soils that did not receive ^{15}N amendments but were treated similarly were otherwise run through the same procedure to allow for the correction of background ^{15}N level for TN_{fixed} calculations.

To distinguish between the (i) $^{15}\text{N}_{\text{fixed}}$ held within the clay lattice, the mineral fraction of soils, and (ii) the $^{15}\text{N}_{\text{fixed}}$ held by the soil organic material, we followed an extraction procedure for soil organic matter as described in detail by Stevenson (1982). We made use of the same soil samples as for the total N_{fixed} determination and subjected the soil to alkali extraction with NaOH. For this purpose, a second set of soil aliquots (100 mg) were weighed into 2 mL Eppendorf tubes, and were treated in the same way as described in determining TN_{fixed} . However, after the washing step with 1.5 mL of ultrapure water, instead of the drying step, we added 0.5 mL 0.5 M NaOH to the soil at a ratio of 1 : 5 (soil : NaOH) as suggested by Wolf et al. (1994b). The soils were then extracted for 18 h (2 h in an ultrasonic bath, 16 h on a shaker). After the extraction period, samples were centrifuged at 1500 x g for 10 min, and 50 μL of the dark brown colored supernatant containing the humic substances was pipetted into tin capsules and stored in a drying oven at 60 °C until dry. The tin capsules were then folded and measured for N content and for N isotopic composition via EA–IRMS. Unlabeled soil samples, also extracted with 0.5 M NaOH, served as natural ^{15}N abundance blanks for calculations. By applying an alkali extraction with NaOH, both humic and fulvic acids were extracted, which were viewed as humic substances as a whole. The extraction efficiency for soil organic matter is about 80 % using this alkali extraction method (Stevenson, 1982). We also calculated the enrichment in ^{15}N and the recovery rate for added $^{15}\text{N}_{\text{fixed}}$ inorganically in clay minerals and $^{15}\text{N}_{\text{fixed}}$ in soil organic matter for both the live and sterile soil samples. After being corrected for extraction efficiency, by subtracting the ^{15}N recovery in humic substances from ^{15}N recovery in total N_{fixed} , we thus could obtain the ^{15}N recovery of ^{15}N fixed by the mineral fraction of the

soil.

2.7. Statistical analyses

To conduct our statistical analyses, we made use of the program R (R Core Team, 2013), using the packages *nmle* (Pinheiro et al., 2016) and *MASS* (Venables and Ripley, 2002). We applied linear models on all variables to test for effects of sterilization, time, and their interaction on the recovery rates of the added tracer in different pools (Schwarz, 2009). Validation of the model was examined using graphical tools. Normality was assessed by plotting histograms and normal QQ plots of residuals, and homogeneity was assessed by plotting the pooled residuals against the fitted values (Schwarz, 2009). Where necessary, our model was refined to account for any unequal variance between levels of explanatory variables (Schwarz, 2009). We determined the significance of fixed effects using single term deletions combined with likelihood ratio tests (LR).

To test for any associations between either incubation time and N content, atom % enrichment in ^{15}N , or recovery rates of ^{15}N in the different N pools investigated, we used Pearson's product moment correlation coefficient (r).

For the determination of significant differences between sample means we applied Tukey's range test. Differences were considered significant when $P < 0.05$. Also, linear regressions were performed to investigate linearity of the process rates.

3. Results

3.1. Enrichment in ^{15}N and recovery of the tracer from different N pools

In both soil types the enrichment in $^{15}\text{N}\text{-NH}_4^+$ was sufficient enough in order to measure the dilution of the ^{15}N labeled NH_4^+ pool with high accuracy. Total recovery of the added ^{15}N tracer from extractable and microbial N pools was achieved in the sterile and live forest soil over the entire incubation time of 48 h (Table 3.1). However, in the sterilized grassland soil we recovered 100 % of the added ^{15}N at 0 h but only 90 % after 48 h. In the live grassland soil we recovered 87 % at 0 h and 60 % of the tracer after 48 h from the combined extractable N and microbial N pools.

Table 3.1.: Recovery of added ^{15}N (%) from the combined extractable N and microbial N pools. Means and standard errors are given for the forest soil and the grassland soil ($n = 3$)

Incubation time (h)	Forest		Grassland	
	Sterile	Live	Sterile	Live
0	112.6 (0.9)	106.5 (0.6)	100 (0.9)	86.9 (1.7)
0.25	110.3 (0.8)	104.5 (0.2)	96.9 (0.2)	60.8 (2.1)
3.5	110.1 (4.2)	103.2 (1.3)	97.4 (0.3)	63.3 (0.2)
24	111.8 (1.0)	100.3 (0.7)	96.8 (1.8)	64.5 (1.4)
48	111.6 (2.1)	98.5 (0.8)	90.2 (4.6)	59.6 (2.3)

In both soils, time and soil treatment (sterilization) had a significant effect on the

3. Results

recovery rate of ^{15}N from the NH_4^+ pool and from the NO_3^- pool (Table 3.2). In the forest soil, the recovery rate of ^{15}N in DON was not significantly affected by the interaction between time and sterilization but rather by the factors individually. However, in the grassland soil, the recovery rate of ^{15}N in DON was neither affected by the interaction between time and sterilization nor by the factors individually (Table 3.2). Our model did not allow for determining the significance of incubation time and treatment on the recovery rate of the tracer from the microbial N pool, as recovery was zero in the sterilized soils over the course of incubation.

For the NH_4^+ pool in the forest soil, the ^{15}N recovery rate was significantly affected by the interaction between incubation time and sterilization ($T \times S$: LR = 15.4, $df = 4,16$, $P = 0.0040$). More specifically, the recovery rate decreased in the live soil by approximately 22 % over the entire incubation time. In the sterile soil, the recovery rate varied between 78 % and 100 % (Fig. 3.1). Recovery rates of ^{15}N from the NO_3^- pool were significantly affected by the interaction between time and sterility ($T \times S$: LR = 81.0, $df = 4,16$, $P < 0.0001$). Moreover, the recovery of the tracer from the NO_3^- pool increased from 0 % (0 h) to 4 % at 24 h and 7.5 % after 48 h. In the sterile soil, the recovery rate remained at between 0.2 % and 0.3 %. In the DON pool, sterilization had an apparent effect on the recovery rate of added ^{15}N , but the pattern over incubation period was similar in sterile and live soil samples. Differences between sterile and live soil were consistent over time.

The interaction between incubation time and sterilization of the grassland soil significantly affected tracer recovery rates from the NH_4^+ pool ($T \times S$: LR = 70.4, $df = 4,15$, $P < 0.0001$) and from the NO_3^- pool ($T \times S$: LR = 77.6, $df = 4,16$, $P < 0.0001$), but not from the DON pool (Fig. 3.1). Specifically, in the NH_4^+ pool, the recovery rate of ^{15}N from the live soil decreased by 97 % over the 48 h incubation time, whereas in the sterile soil, recovery rates decreased only by approximately 12 %. In contrast, the recovery rate of ^{15}N from the NO_3^- pool increased significantly in the live soil. After 48 h, 52 % of the added tracer was recovered from the NO_3^- pool. The recovery rates from the sterilized soil samples did not, however, change

over time for the NO_3^- pool. We did not observe any changes in the recovery rate of ^{15}N from DON ($T \times S$: $\text{LR} = 2.24\text{e-}05$, $df = 4,15$, $P = 1$) over incubation time, neither in the live nor in the sterile soil samples. Differences in recovery rates from the DON pool between the live and sterile soil samples were only marginal at all times.

Conclusions on the recovery rate from the N_{mic} pool could not be drawn using our statistical model but are examined in section 3.2 using non-parametric tests.

Table 3.2.: Significance of interaction between sterility and time ($S \times T$) and significance of sterility (S) and time (T) individually on recovery rates of ^{15}N (%) from different N pools (NH_4^+ , NO_3^- , DON) in the forest soil and the grassland soil. Values are given for the likelihood ratio test (LR), the degrees of freedom (df), and the significance level of the interaction or the individual term on the recovery rate of ^{15}N . Asterisks indicate the significance of the interaction of variables ($S \times T$) or the significance of a single variable (S , T) on the recovery rate of ^{15}N (significance codes $P < 0$ '****' 0.001 '***' 0.01 '**')

N pool	factor	Forest			Grassland		
		LR	df	P	LR	df	P
NH_4^+	$T \times S$	15.4	4,16	0.0040 **	70.4	4,15	< 0.0001 ***
	T	23.1	4,12	0.0001 ***	22.0	4,11	0.0002 **
	S	11.7	1,12	0.0006 ***	94.3	1,11	< 0.0001 ***
NO_3^-	$T \times S$	81.0	4,16	< 0.0001 ***	77.6	4,16	< 0.0001 ***
	T	5.9	4,12	0.2047	13.2	4,12	0.0106 *
	S	3.6	1,12	0.0587	18.9	1,12	< 0.0001 ***
DON	$T \times S$	4.7	4,16	0.3211	2.2e-05	4,15	1.0000
	T	36.2	4,12	< 0.0001 ***	3.4e-05	4,11	1.0000
	S	20.0	4,12	< 0.0001 ***	4.9e-06	1,11	0.9982

The total recovery of the added tracer from the live grassland soil was observed for the incubation periods 0 h and 24 h by analyzing the TN_{fixed} pool (Table 3.3). We recovered 24 % of the added tracer from the live soil immediately after tracer addition (0 h) and 38 % after 24 h. At 0 h, 100 % of the fixed ^{15}N was held within the inorganic fraction of the soil. After 24 h of incubation, 97 % of the fixed ^{15}N

3. Results

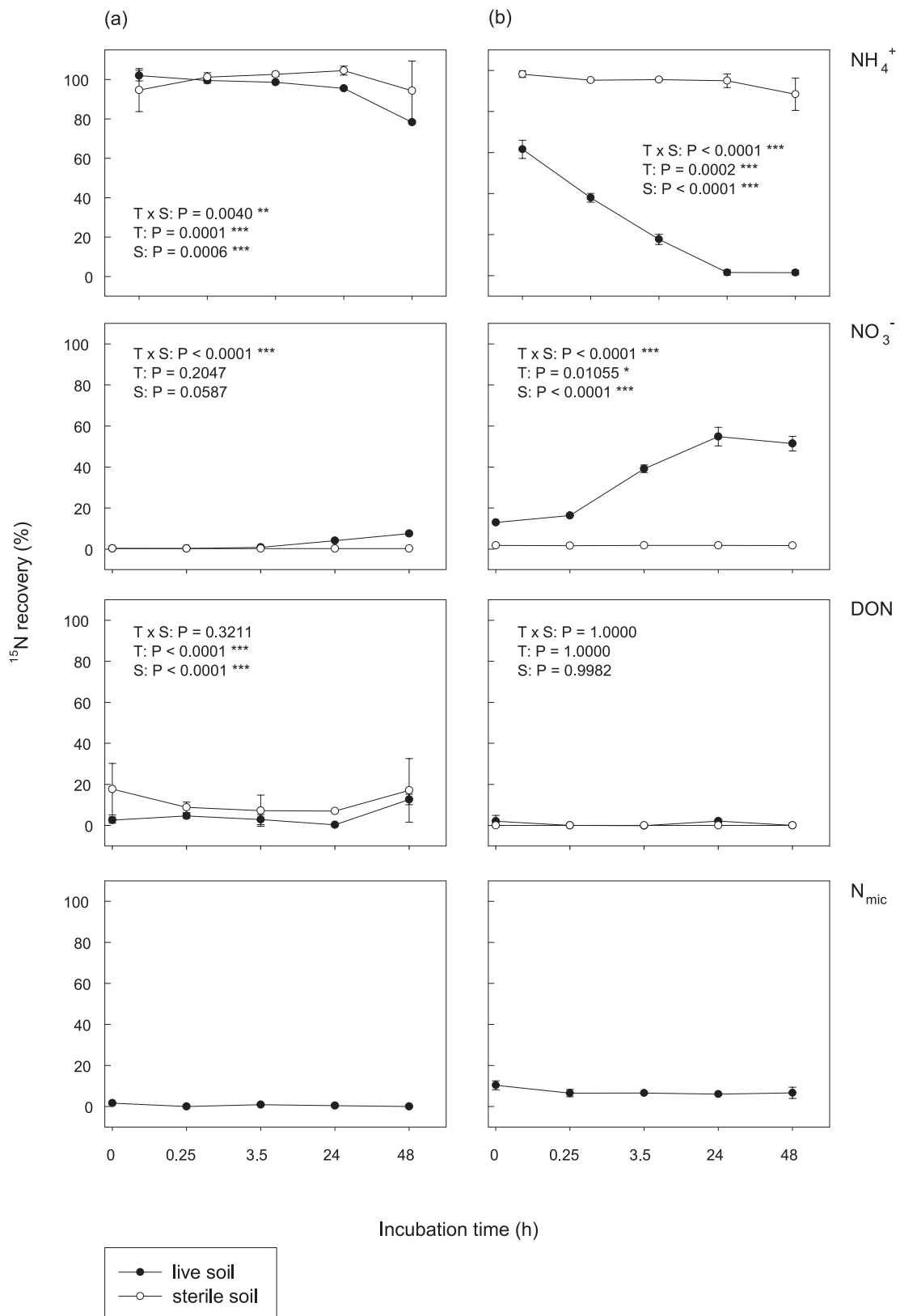


Figure 3.1.: Mean recovery rates (% $\pm$ 1 SE) of ^{15}N from different N pools (NH_4^+ , NO_3^- , DON, N_{mic}) in the live and sterile forest soils (a) and grassland soils (b) at 0 h, 0.25 h, 3.5 h, 24 h, and 48 h

was recovered from the inorganically fixed fraction and 3 % from the organically fixed fraction (Fig. 3.2). The change in recovery rate of ^{15}N from the fixed N pool was significant over time in both the fraction fixed by clay minerals ($P=0.0155$) as well as in the fraction fixed by humic substances ($P=0.0280$).

When analyzing the recovery rate from TN_{fixed} in the live forest soil, we calculated a mean recovery rate of 1.8 % for the ^{15}N tracer at 0 h and 15.4 % at 24 h. The recovery rate of added ^{15}N increased significantly over incubation time from 0 h to 24 h in the inorganically fixed fraction ($P=0.0008$) but remained less than 1 % in the organically fixed fraction ($P=0.3010$) during incubation time up to 24 h (Fig. 3.2).

For the sterilized soil samples, only the recovery rate of added ^{15}N from the TN_{fixed} fraction for the samples extracted after 24 h was estimated (data not shown). In the sterilized forest soil samples, we recovered 2 % of the added ^{15}N from the inorganic fraction. However, in the sterilized grassland soil samples, we recovered 18 % of the tracer from the inorganic fraction of the fixed N. In both sterilized soils, no tracer was bound to humic substances after 24 h. The recovery rate of fixed ^{15}N was significantly higher in the live soils when compared to the sterile soils (Forest: $P=0.0020$; Grassland: $P=0.0083$).

Ultimately, we were able to recover all the tracer added to the soil at two incubation periods, in which we measured all N pools, 0 h and 24 h (see Table 3.3).

Table 3.3.: Contribution of the investigated N pools (%) as sinks for added $^{15}\text{N}-\text{NH}_4^+$ in live forest (F) and live grassland soils (G) during the IPD experiment at incubation time 0 h and 24 h. Means and standard errors are given ($n = 3$)

Soil	time (h)	NH_4^+	NO_3^-	DON	N_{mic}	N_{fixed}	Sum
F	0	102 (1.5)	0.3 (0.0)	2.6 (0.8)	1.6 (0.2)	1.8 (0.4)	108.3 (10.7)
	24	95.5 (0.4)	4.1 (0.0)	0.3 (0.3)	0.4 (0.4)	15.4 (1.0)	115.7 (9.8)
G	0	61.6 (2.5)	13 (0.1)	2 (0.6)	10.3 (1.3)	24 (2.7)	110.9 (5.6)
	24	1.5 (0.7)	54.9 (2.6)	2.1 (0.4)	6 (0.4)	38 (0.5)	102.5 (5.9)

3. Results

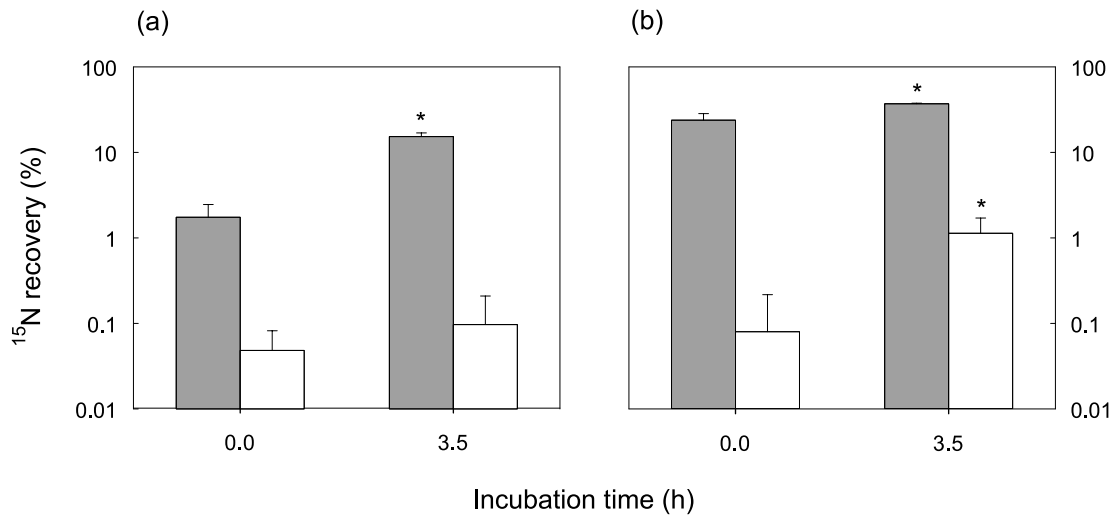


Figure 3.2.: Logarithmic mean recovery rates of added ^{15}N from the inorganically fixed N fraction (shaded bars) and the organically fixed N fraction (white bars) in (a) live forest soil and (b) live grassland soil (% , ± 1 SE) at sampling dates 0 h and 24 h ($n = 3$). Significant differences between the rates at 0 h and 24 h in (a) and (b) are indicated by an *asterisk* (Tukey: $P < 0.05$)

3.2. Assessment of ^{15}N reflux sources and the importance of eventual reflux

Correlations between incubation time and the recovery rates in the live soils revealed that the amount of ^{15}N recovered either increased significantly over incubation time in the NH_4^+ sinks (Forest soil: NO_3^- , DON, N_{fixed} ; Grassland soil: NO_3^- , N_{fixed}) or did not show a clear trend, i.e. in the DON in the grassland soil (Table 3.4). Only in the N_{mic} pool, a slight decrease of the recovery rates of added ^{15}N was observed in both soils. However, the decrease in recovery rates of added ^{15}N was not significant over the incubation period of forty–eight hours (Forest soil: $P = 0.064$; Grassland soil: $P = 0.2944$) (Table 3.4).

Therefore, we identified N_{mic} as the only possible source for the reflux of immobilized labeled NH_4^+ to the available ammonium pool over incubation time.

The possible impact of a reflux from the N_{mic} pool on the NH_4^+ gross production

Table 3.4.: Significance of the change in ^{15}N recovery (%) of the added $^{15}\text{N}-\text{NH}_4^+$ over incubation time in the different N pools measured in the live forest soil (F) and the live grassland soil (G). Significance was tested using Pearson's product-moment correlation (r). Change in ^{15}N recovery over the incubation time of 48 hours was considered significant when $P < 0.05$

N pool	Forest		Grassland	
	r	P	r	P
NH_4^+	-0.94	< 0.0001	-0.94	< 0.0001
NO_3^-	0.96	< 0.0001	0.91	< 0.0001
DON	0.66	0.0075	0.00	< 0.0001
N_{mic}	-0.49	0.0647	-0.29	0.2944
N_{fix}	0.99	0.0002	0.93	0.0071

rate was investigated in both soils using sensitivity analysis. Reflux rates of $^{15}\text{N}-\text{NH}_4^+$ of 10 %, 20 %, 50 %, and 100 % were simulated for the incubation period from 3.5 h to 24 h, which is considered to be a usual incubation time during isotope pool dilution experiments (Murphy et al., 2003).

The initial NH_4^+ concentrations and APE at t_0 (3.5 h) were constant, but NH_4^+ concentrations and APE at t_1 (24 h) were recalculated for the different scenarios, which will be described below.

In more detail, we simulated a reflux for the amount of $^{15}\text{N}-\text{N}_{\text{mic}}$, which was rapidly taken up by microbes during the first 15 min of incubation time. Therefore, the APE of $^{15}\text{N}-\text{NH}_4^+$ for the mean atom % enrichment in the initial incubation phase (APE at $t_1 = \bar{x}_{\text{APE at } 0 \text{ h}, 0.25 \text{ h}}$) needed to be recalculated. The tracer reflux was calculated at increasing rates of 10 %, 20 %, 50 %, and 100 %. In order to correct the APE of $^{15}\text{N}-\text{NH}_4^+$ at t_1 for the $^{15}\text{N}-\text{N}_{\text{mic}}$ reflux, we added the average amount of $^{15}\text{N}-\text{N}_{\text{mic}}$ in excess at 3.5 h and 24 h to the amount of $^{15}\text{N}-\text{NH}_4^+$ in excess at 24 h and recalculated it back to the APE $^{15}\text{N}-\text{NH}_4^+$. 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 percent enrichment of the NH_4^+ during the initial incubation phase (0 h–0.25 h), and the average amount

3. Results

of ^{15}N in excess calculated for t1, we were able to estimate the amount of NH_4^+ feeding back into the available ammonium pool concomitant with the respective amount of $^{15}\text{N}-\text{NH}_4^+$.

We subsequently estimated the gross production rates of NH_4^+ for assessing the importance of an eventual reflux of labeled NH_4^+ taken up by microbes in the available ammonium pool. In the forest soil, the reflux caused only a modest underestimation of NH_4^+ gross production rates. At a reflux rate of 10 %, the NH_4^+ gross production rate was not affected at all (Table 3.5). In contrast, a simulated worst case scenario for the grassland soil revealed an underestimation of the NH_4^+ gross production rate by up to 63 %. At a reflux rate of 50 %, the rate was still underestimated by 43 % and 14 % at a reflux rate of 10 % of the labeled NH_4^+ that was taken up by microbes (Table 3.5).

Table 3.5.: Sensitivity analysis of the effect of $^{15}\text{N}-\text{NH}_4^+$ reflux at different rates (10 %, 20 %, 50 %, and 100 %) from the N_{mic} pool on the mean gross mineralization rate (GP) ($\mu\text{g N g}^{-1}$ soil dry weight d^{-1} , ± 1 SE) between incubation time 3.5 h and 24 h simulated for the forest soil (F) and the grassland soil (G) ($n = 3$)

^{15}N reflux (%)	Forest GP ($\mu\text{g N g}^{-1} \text{d}^{-1}$)	Difference (%)	Grassland GP ($\mu\text{g N g}^{-1} \text{d}^{-1}$)	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

3.3. Linearity of NH_4^+ transformation rates

Our results proved that transformation processes of NH_4^+ occurred at constant rates over a certain period of incubation in the live forest and grassland soils. The natural logarithm of atom percent excess of ^{15}N was plotted against incubation time to test for linearity (Fig. 3.3). In the forest soil, we found transformation

process rates to be constant between 3.5 h and 48 h ($R^2 = 0.979$). However, in the grassland soil, rates were constant between 15 min (0.25 h) and 24 h of incubation ($R^2 = 0.904$). The atom percent ^{15}N decreased faster in the grassland soil ($k = -0.128$) as compared to the forest soil ($k = -0.003$) in the respective incubation period plotted (Fig. 3.3).

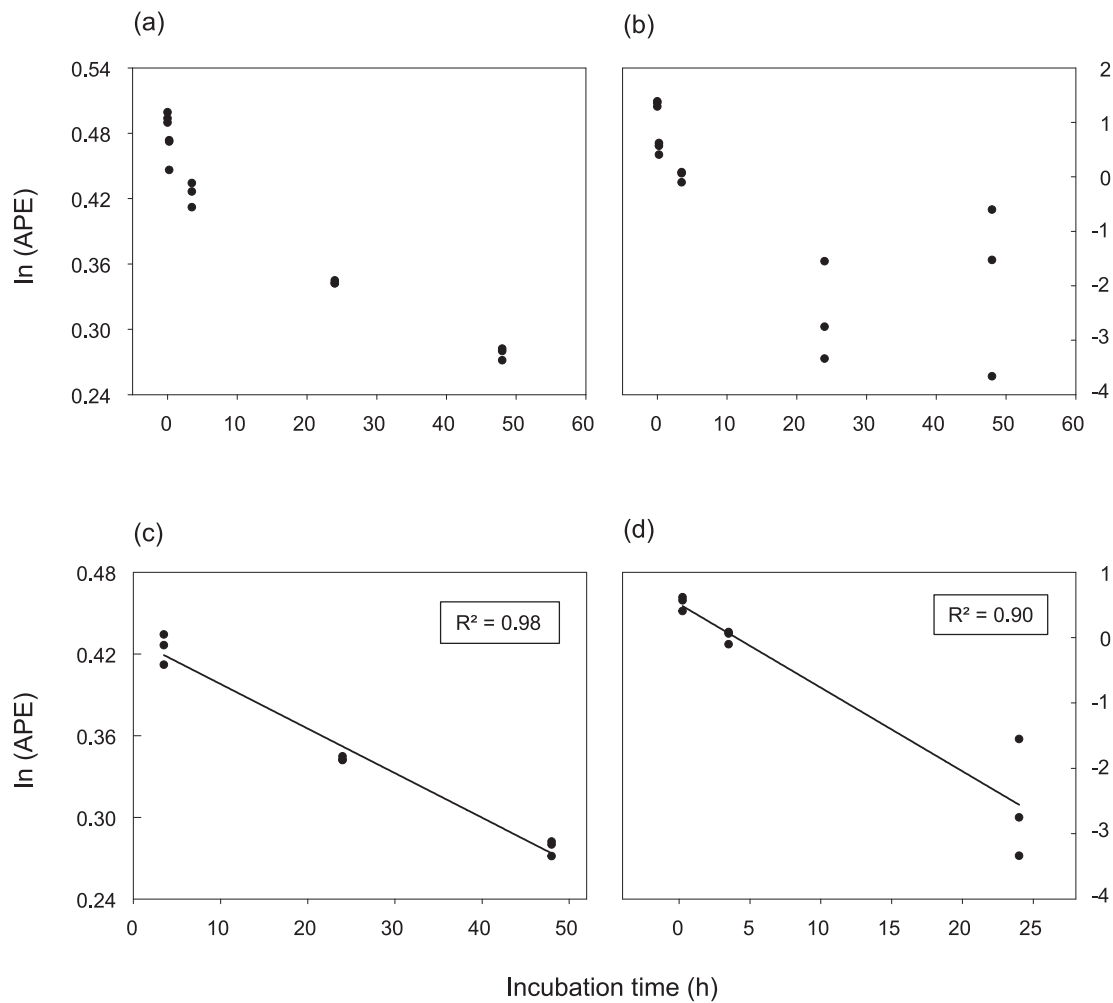


Figure 3.3.: Correlation between logarithmic atomic percent excess (APE) of $^{15}\text{N}-\text{NH}_4^+$ in live forest soils (a) over the total incubation time (0 h to 48 h) and (c) over incubation time 3.5 h to 48 h and in live grassland soils (b) over total incubation time (0 h to 48 h) and (d) over incubation time 0.25 h to 24 h ($n = 3$). The regressions in (c) and (d) are based on APE estimations at three time points in the forest soil ($k = -0.003$, $R^2 = 0.98$, $P < 0.0001$) and in the grassland soil ($k = -0.128$, $R^2 = 0.90$, $P < 0.0001$)

3.4. Gross transformation rates of NH_4^+

In our calculation of NH_4^+ gross production and gross consumption rates for live forest and grassland soils for the time interval 3.5 h–24 h, process rates were shown to be constant in both soils. Our results further revealed significant differences in process rates between the two soils (Fig. 3.4). Gross production was significantly higher in the grassland soil ($P=0.0084$), as was the gross consumption rate ($P=0.0099$). Gross production and consumption rates estimated for the 3.5 h to 24 h period were significantly different from each other in the forest soil ($P=0.0174$). This was not the case in the gross rates in the grassland soil ($P=0.1841$).

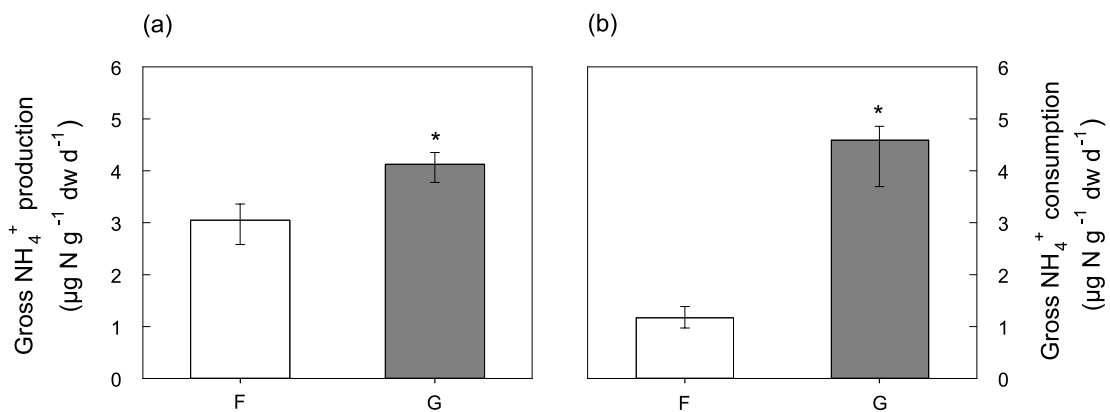


Figure 3.4.: Mean gross production rates (a) and gross consumption rates (b) of NH_4^+ ($\mu\text{g N g}^{-1} \text{ soil dry weight d}^{-1} \pm 1 \text{ SE}$) in live forest (F) and grassland (G) soils for the time interval 3.5 h–24 h ($n=3$). Significant differences between soil types are indicated by an *asterisk* (Tukey: $P < 0.05$)

4. Discussion

The aim of the study was to determine the fate of $^{15}\text{N}\text{-NH}_4^+$ tracer during a short-term IPD experiment performed on two soils, one of them consuming NH_4^+ very rapidly. We wanted to assess the main sink pathways and whether a reflux of consumed ^{15}N tracer into the available ammonium pool is likely for any of the identified NH_4^+ sinks during incubation time. A reflux of tracer may have a large impact on the estimation of gross N transformation rates, especially for the rapidly consuming grassland soil. Therefore, we evaluated its importance for gross N mineralization estimates in the soils investigated.

4.1. Recovery of the $^{15}\text{N}\text{-NH}_4^+$ tracer

Our measurements revealed the fate of the NH_4^+ over incubation time in both soils. For the live forest soil, we found that the nitrification of NH_4^+ immobilized the tracer to a small extent (8 % after 48 h) and microbial uptake accounted for only 1.6 % of the tracer recovery immediately after tracer addition, which decreased over time to 0 % after 48 hours (Fig. 3.1). The remaining consumed tracer was recovered from the DON pool (13 % after 48 h). After 24 hours, 15 % of the tracer was abiotically fixed. The biggest proportion (78 % after 48 h) remained in fact as $^{15}\text{N}\text{-NH}_4^+$ and was able to be extracted from the soil. Consumption rates of the ^{15}N tracer proved rather slow in the forest soil.

In contrast, the $^{15}\text{N}\text{-NH}_4^+$ pool in the live grassland soil was depleted by the end

4. Discussion

of the 48 hours incubation period (Fig. 3.1). After two days of incubation, nitrification made up for the biggest proportion of tracer consumption (> 50 %). We found that the nitrification of the $^{15}\text{N}-\text{NH}_4^+$ set in immediately after tracer addition. The same applied for the microbial uptake of NH_4^+ . Both biotic processes combined were responsible for 23 % of the tracer loss within minutes after $^{15}\text{N}-\text{NH}_4^+$ addition. Complementing the tracer recovery, another 24 % of the added tracer was recovered from the fixed ammonium pool immediately after tracer addition (Table 3.3). These findings clearly show that the rapid consumption of NH_4^+ , as observed in the live grassland soil, derive from both processes: biotic immobilization and abiotic fixation.

Many studies reported that biotic processes are considered to be the main factor responsible for rapid $^{15}\text{N}-\text{NH}_4^+$ consumption in isotope pool dilution and tracer immobilization studies in different soils (Fitzhugh et al., 2003; Bruun et al., 2006; Herrmann et al., 2007; Hill et al., 2012; Wilkinson et al., 2014). Also, the rapid abiotic fixation of $^{15}\text{N}-\text{NH}_4^+$ has been reported for various soil types from isotope dilution assays in combination with sterilization experiments (Kowalenko and Cameron, 1978; Davidson et al., 1991). Other studies highlighted both biotic immobilization and abiotic fixation responsible for rapid $^{15}\text{N}-\text{NH}_4^+$ consumption (Schimel and Firestone, 1989; Trehan, 1996; Johnson et al., 2000; Morier et al., 2008). To which extent one or the other process prevails depends on many factors, among them the C and N content of the soil (Booth et al., 2005), soil moisture (Gouveia and Eudoxie, 2007), NH_4^+ fixation capacity, and clay content and composition (Nieder et al., 2011).

We found that when compared to the forest soil, a higher recovery rate of abiotically fixed $^{15}\text{N}-\text{NH}_4^+$ in the grassland soil. This may have resulted from a higher NH_4^+ fixation capacity in the grassland soil due to a higher clay content when compared to the forest soil (clay content: Grassland: 26 %; Forest: 16 %). The higher NH_4^+ fixation capacity, in combination with the lower initial NH_4^+ concentration in the grassland soil, could be the reason for the more rapid abiotic fixation of NH_4^+

and the higher rate at which the tracer was fixed. Davidson et al. (1991) reported similar findings on the importance of abiotic reactions as sinks for $^{15}\text{N}-\text{NH}_4^+$ in both forest and grassland soil.

Measurements in the sterilized soil samples confirm our findings from the live soils (data not shown). Abiotic fixation of NH_4^+ was observed in the sterile soils after 24 hours of incubation with $^{15}\text{N}-\text{NH}_4^+$. Though, in the forest soil, we found abiotic fixation to be only very little in the sterile soil (2 % recovery) when compared to the live soil (15 % recovery). Similarly, in the sterilized grassland soil, the recovery rate of the tracer after 24 hours was only 18 % compared to 38 % tracer recovery in the live soil. Autoclaving is known to increase the amount of NH_4^+ significantly in soil (Lopes and Wollum, 1976; Wolf and Skipper, 1994). In accordance with that, we found the extractable NH_4^+ concentration in the sterilized forest soil to be almost four times as high as when compared to the live soil. In the grassland soil, autoclaving increased the amount of NH_4^+ even by a factor of 27 (Appendix, Fig. 1.1). Thus, competition for the cation binding sites in the clay mineral interlayers was significantly increased, and consequently, $^{15}\text{N}-\text{NH}_4^+$ was less likely to become bound. Also, Trehan (1996) reports that fixation by clay is slightly greater in non-autoclaved soils when compared to autoclaved soils. Trehan suggested that autoclaving changes the structure of the clay to a small extent, thus reducing the number of sites for NH_4^+ fixation. This might also apply to a certain extent in our experiment, although autoclaving is not assumed to change the surface area of clay minerals (Wolf and Skipper, 1994).

4.2. Reflux evaluation of the abiotic sinks for NH_4^+

According to the recovery rates of the $^{15}\text{N}-\text{NH}_4^+$ tracer, in this experiment, we found that abiotic fixation of ammonium was, in both soils, almost exclusively due to the mineral fraction of the soil (Fig. 3.2).

4. Discussion

In general, a release of fixed $^{15}\text{N}\text{-NH}_4^+$ 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 (Nõmmik, 1965; Kowalenko and Cameron, 1978; Steffens and Sparks, 1997). The release of fixed NH_4^+ is, therefore, mainly depending on the NH_4^+ concentration in the solution phase. In our study, we did not observe a significant decrease in the total NH_4^+ pool over incubation time in the forest soil (Appendix, Table 1.1). Therefore, we concluded that re-diffusion of fixed $^{15}\text{N}\text{-NH}_4^+$ into the available pool is highly unlikely. In contrast, a significant decrease in the total available NH_4^+ content was observed over incubation time in the grassland soil (Appendix, Table 1.1). However, the constant increase of the ^{15}N recovery rate from the clay fixed N pool is arguing against a reflux of tracer from clay interlayers. Besides, this finding is supported by other studies that demonstrated the release kinetics of clay fixed NH_4^+ into soil solution range from weeks to years (Kowalenko and Cameron, 1978; Nieder et al., 2011).

Therefore, we concluded that a reflux of abiotically clay fixed ^{15}N did not occur in our experiment for both soils. On the contrary, the clay fixation of $^{15}\text{N}\text{-NH}_4^+$ could be concomitant with the release of native fixed NH_4^+ . In this case, the rates of gross N mineralization could be slightly overestimated. However, in long-term IPD experiments, the reflux of fixed $^{15}\text{N}\text{-NH}_4^+$ needs to be considered, especially since recently fixed NH_4^+ is assumed to be more likely to be released from the clay lattice than native NH_4^+ (Nieder et al., 2011).

The major fraction of abiotic fixation in our experiment was due to fixation by the mineral fraction of the soil. Nevertheless, in the grassland soil, we found a small fraction of the abiotically fixed $^{15}\text{N}\text{-NH}_4^+$ being bound to the organic fraction of the soil (3 % after 24 hours). 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 physical condensation reactions of phenolic hydroxyls, hydroquinones and quinone polymers with NH_3 (Burge and Broadbent, 1961;

Stevenson, 1982; Nõmmik and Vahtras, 1982; Thorn and Mikita, 1992). However, covalent bonding of ammonia to soil organic matter is supposed to result in fairly stable compounds that are only slowly mineralized by soil microorganisms (Thorn and Mikita, 1992; Monaghan and Barraclough, 1995). Our findings on the contribution of the mineral and the organic fraction to NH_4^+ fixation are consistent with the results of other studies (Kowalenko and Cameron, 1978; Nõmmik and Vahtras, 1982; Trehan, 1996). Since bonding to humic substances only happens to a small extent, and degradation is supposed to be slow, we assume that there is no need to consider the remineralization of organically fixed $^{15}\text{N}-\text{NH}_4^+$ as a source for reflux in this study.

Interestingly, in the forest soil, the recovery rate of ^{15}N from the DON pool was significantly affected by both sterilization and incubation time (Table 3.2). The recovery rate increased significantly over time (Appendix, Table 1.3). However, our results on DON could well be due to experimental errors since this data does not refer to direct DON measurements but is based on TDN, NH_4^+ , and NO_3^- measurements. 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 resulting from microbial turnover (Seely and Lajtha, 1997) or microbial production of extracellular enzymes (Trasar-Cepeda et al., 2000) can also be excluded in our experiment due to the low amount of $^{15}\text{N}-\text{NH}_4^+$ taken up by microbes in the forest soil (Table 3.3).

However, abiotic fixation by the organic fraction of the soil, similar to bonding with humic substances as described above, could also be an explanation for the increased ^{15}N tracer recovery in DON. An argument against the covalent bonding of the labeled NH_4^+ would be the low pH in the forest soil (pH 4). Though, covalent bonding of NH_4^+ to organic compounds has been only reported in the form of NH_3 , which is usually forming in soil under alkaline conditions (Burge and Broadbent, 1961; Thorn and Mikita, 1992). So far, there are only a few studies looking into the biodegradability of DON, and its full ecological significance is not well under-

stood yet (Jones et al., 2004). However, Jones et al. (2004) suggested that a part of the DON, low-molecular weight compounds, 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 TDN in soil (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; Wilkinson et al., 2014). Therefore, it might be interesting for other studies to also investigate DON mineralization, when considering a reflux of tracer to the available NH_4^+ pool, especially in long-term IPD experiments.

4.3. Reflux evaluation of the biotic sinks for NH_4^+

Effectively, we found that only the recovery rate of ^{15}N from the microbial N pool decreased slightly over the incubation period of 24 hours (Fig. 3.1, Table 3.4).

The sensitivity analysis of the reflux of microbially taken up $^{15}\text{N}\text{-NH}_4^+$ to the ammonium pool simulated for a short-term incubation of 24 hours revealed only a minor impact in the forest soil (Table 3.5). According to our simulations, the gross N mineralization rate could be underestimated by a maximum of only 3 %. In contrast, the gross N mineralization rate in the grassland soil could be underestimated in the worst case scenario by up to 63 % (Table 3.5).

The low impact of simulated microbial N reflux in the forest soil can be most likely explained by the low amount of $^{15}\text{N}\text{-NH}_4^+$ taken up and the little contribution of this amount to the initial high NH_4^+ concentration. However, in the grassland soil, a reflux of the high amount of $^{15}\text{N}\text{-NH}_4^+$ taken up by microbes, combined with the low NH_4^+ concentration in the soil, had a large impact on the estimation of gross NH_4^+ N transformation rates.

However, such an extent of reflux of ^{15}N taken up by microbes in the grassland soil seems unlikely during an experimental period of 24 hours. Fast NH_4^+ efflux

due to leakage from microbial cells is concomitant with the uptake or influx of NH_4^+ and could have occurred (Morgan and Jackson, 1988; Ludewig et al., 2007). Even active channel- or carrier-mediated NH_4^+ transport out of cells is possible but we assume that the amount of ^{15}N efflux is minimal under the stable conditions of our experiment (Hadas et al., 1992; von Wirén and Merrick, 2004). This is especially true for the grassland soil due to its low NH_4^+ concentration (Bengtsson and Bengtsson, 2005). In contrast, re-mineralization, the mineralization of previously immobilized and assimilated N, would be a much slower process than a NH_4^+ efflux. But a re-mineralization of ^{15}N could represent a source for tracer reflux in the grassland soil, at rates impacting the gross N mineralization rate.

The rapid incorporation of $^{15}\text{N}\text{-NH}_4^+$ into the biomass could explain the decrease in ^{15}N enrichment and in recovery from the microbial N pool. In fact, the microbial biomass content, at least for the grassland soil, increased significantly over incubation time (Appendix, Table 1.1). It is impossible to extract the total amount of ^{15}N taken up by microbes with the sCFE extraction applied in this experiment, if $^{15}\text{N}\text{-NH}_4^+$ became metabolized and built in into cell walls (Fierer and Schimel, 2003). In general, the application of chloroform extraction methods only enables the detection of soluble N compounds within microbial cells, not insoluble ones such as cell wall proteins or peptidoglycans (Jenkinson et al., 2004). This means we would be facing a continuous uptake of NH_4^+ from soil solution, in combination with the ongoing removal from the extractable N_{mic} pool as microbes metabolize the compound. This would ultimately result in a decrease in recovery rate of ^{15}N from the N_{mic} pool.

Usually, turnover of microbial N and its re-mineralization is assumed to take a few days (McGill et al., 1975; Herman et al., 2006), which means that in a short-term laboratory incubation of 24 hours as applied in our IPD experiment, re-mineralization of assimilated $^{15}\text{N}\text{-NH}_4^+$ is relatively unlikely. Therefore, our results show that the reflux of recently fixed ^{15}N tracer from the microbial N pool could have a large impact on the estimation of gross N mineralization rates, but

it appears to be unlikely during incubation periods of about 24 hours.

These findings are in line with other studies, for example Bengtson and Bengtson (2005), showed that in isotope pool dilution experiments, re-mineralization is lowest during the first two days of incubation. Also Davidson et al. 1991, Herrmann et al. 2007, and others (Bjarnason, 1988; Barraclough, 1995; Murphy et al., 2003; Wang et al., 2001) found that re-mineralization is negligible in IPD experiments during incubation times between 24 hours and up to a few days, even in studies on rapidly immobilizing grassland soils (Davidson et al., 1991).

4.4. Linearity of NH_4^+ transformation rates

Since Kirkham and Bartholomew (1954) assumed constant process rates for estimating gross N transformation, we also investigated linearity of the transformation rates over time. We found gross N mineralization rates to be constant in the two different soils at varying time intervals (Fig. 3.3). For the forest soil, transformation rates seemed to be linear from 3.5 hours after label addition until up to 48 hours of incubation. In the grassland soil, process rates appeared to be linear after fifteen minutes of incubation until up to 24 hours. Gross N mineralization rates calculated for all the time intervals decreased with increasing incubation time (data not shown). This finding is consistent with other studies and can be explained by considering the time needed for the equilibration of the ^{15}N with the ^{14}N pool (Bjarnason, 1988; Watson et al., 2000). This refers to another key assumption of the IPD approach, namely that tracer and tracee behave in the same way in soils (Kirkham and Bartholomew, 1954). The different time intervals appropriate for the estimation of gross N mineralization rates for the two investigated soils underline the importance of preliminary studies of the isotope mixing and consumptive processes when applying the isotope pool dilution technique. Especially for soils showing rapid consumption of NH_4^+ and high turnover rates of inorganic N, it is important to find a balance between (i) the initial time needed to

achieve tracer equilibrium with the native pool (and thereby achieving an identical behavior of the tracer and the tracee) and (ii) the amount of depletion of the ^{15}N pool. As in most soil NH_4^+ pools, the grassland $^{15}\text{N}-\text{NH}_4^+$ was almost fully depleted after only 24 hours (Booth et al., 2005). This time frame does not allow for an equilibration time of 24 hours before initial sampling as recommended by many authors (Watson et al., 2000; Cliff et al., 2002; Murphy et al., 2003; Herrmann et al., 2007). In the case of a 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 prolong incubation time (Murphy et al., 2003; Herrmann et al., 2007). This has proven to be a good solution for soils showing high nitrification potential (Herrmann et al., 2007) but does not account for 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. However, in our study, a uniform mixing of the tracer solution with the soil was assumed to be achieved by a thorough mixing process (Barraclough, 1995; Di et al., 2000), and the equilibrium of tracer and tracee seemed to be reached after an incubation time of only a few hours. 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 be prolonged, since errors become more significant as ^{15}N enrichment close to natural abundance levels (0.3663%) are approached (Davidson et al., 1991).

4.5. Gross transformation rates of NH_4^+

The estimated gross N mineralization and NH_4^+ consumption rates for the incubation interval between 3.5 h and 24 h reflect the different N turnover rates of these soils. We found higher gross N mineralization rates in the grassland soil

4. Discussion

($4 \mu\text{g N g}^{-1}$ soil dry weight d^{-1}) compared with the forest soil ($3 \mu\text{g N g}^{-1}$ soil dry weight d^{-1}) (Fig. 3.4). Our observations are in accordance with the finding that gross rates of N mineralization are higher in grassland soils when compared to forest soils with similar soil C concentrations (Booth et al., 2005). The faster and higher turnover rates of NH_4^+ in the grassland soil might be the result of (i) a combination of higher NH_4^+ production rate, (ii) the higher nitrification rate due to the lower NH_4^+ concentration in the soil, (iii) a more active microbial community as well as (iv) a higher abiotic fixation rate due to the higher clay content in the soil.

5. Conclusion

Overall, we found that biotic immobilization and abiotic fixation are responsible for the rapid consumption of $^{15}\text{N-NH}_4^+$ in the investigated grassland soil. We found that rapid abiotic fixation is mainly mediated by the mineral fraction of the soil. A reflux of added $^{15}\text{N-NH}_4^+$ was proved relatively unlikely during our short-term laboratory IPD assay of 24 hours and probably also up to 48 hours, which confirms earlier findings of other experiments. But one should keep in mind, as Wang et al. (2001) pointed out, 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 also the appropriate approach for estimating gross N mineralization, either an analytical solution, a numerical approach or a combination of both ^{15}N tracing studies and analyses via process-based models (Wessel and Tietema, 1992; Cliff et al., 2002; Rütting et al., 2011; Andresen et al., 2015).

Applying the isotope pool dilution method implies numerous sources of errors starting with soil disturbance at the beginning of the experiment, to possible non-uniform exploitations of the nutrient pool or even analytical errors, to name a few of them. Referring to tracer reflux, Bjarnason (1988) assumed that, in short-term experiments, “underestimation of transformation rates caused by ignoring re-mineralization is largely cancelled out by the overestimation caused by the constant rate approach”. It is thus impossible to avoid all sources of errors but one

5. Conclusion

should be aware of that they may occur when performing isotope pool dilution experiments.

List of Figures

2.1. Experimental set-up	38
3.1. ^{15}N tracer recovery from NH_4^+ , NO_3^- , DON, and N_{mic} pools	54
3.2. ^{15}N tracer recovery from the fixed N pool	56
3.3. Linearity of NH_4^+ transformation rates	59
3.4. Gross rates of NH_4^+ production and consumption	60
1.1. Change in N contents over incubation time	90
1.2. Change in mean enrichment in ^{15}N in N pools over incubation time	92
1.3. Change in mean recovery rates of added ^{15}N in N pools over incubation time	94

List of Tables

2.1. General soil characteristics	36
2.2. Ammonium specific soil characteristics	36
3.1. ^{15}N tracer recovery from extractable and microbial N pools	51
3.2. Effect of sterility and time on ^{15}N recovery rates; parametric test	53
3.3. Sub-pool contribution to $^{15}\text{N}\text{--NH}_4^+$ tracer consumption	55
3.4. Effect of time on ^{15}N recovery rates; non-parametric test	57
3.5. Sensitivity analysis of $^{15}\text{N}\text{--NH}_4^+$ reflux	58
1.1. Significance of change in N contents over incubation time	91
1.2. Significance of change in mean enrichment in ^{15}N in N pools over incubation time	91
1.3. Significance of change in mean recovery rates of added ^{15}N in N pools over incubation time	93

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Part III.

Appendix

1. Supplementary material

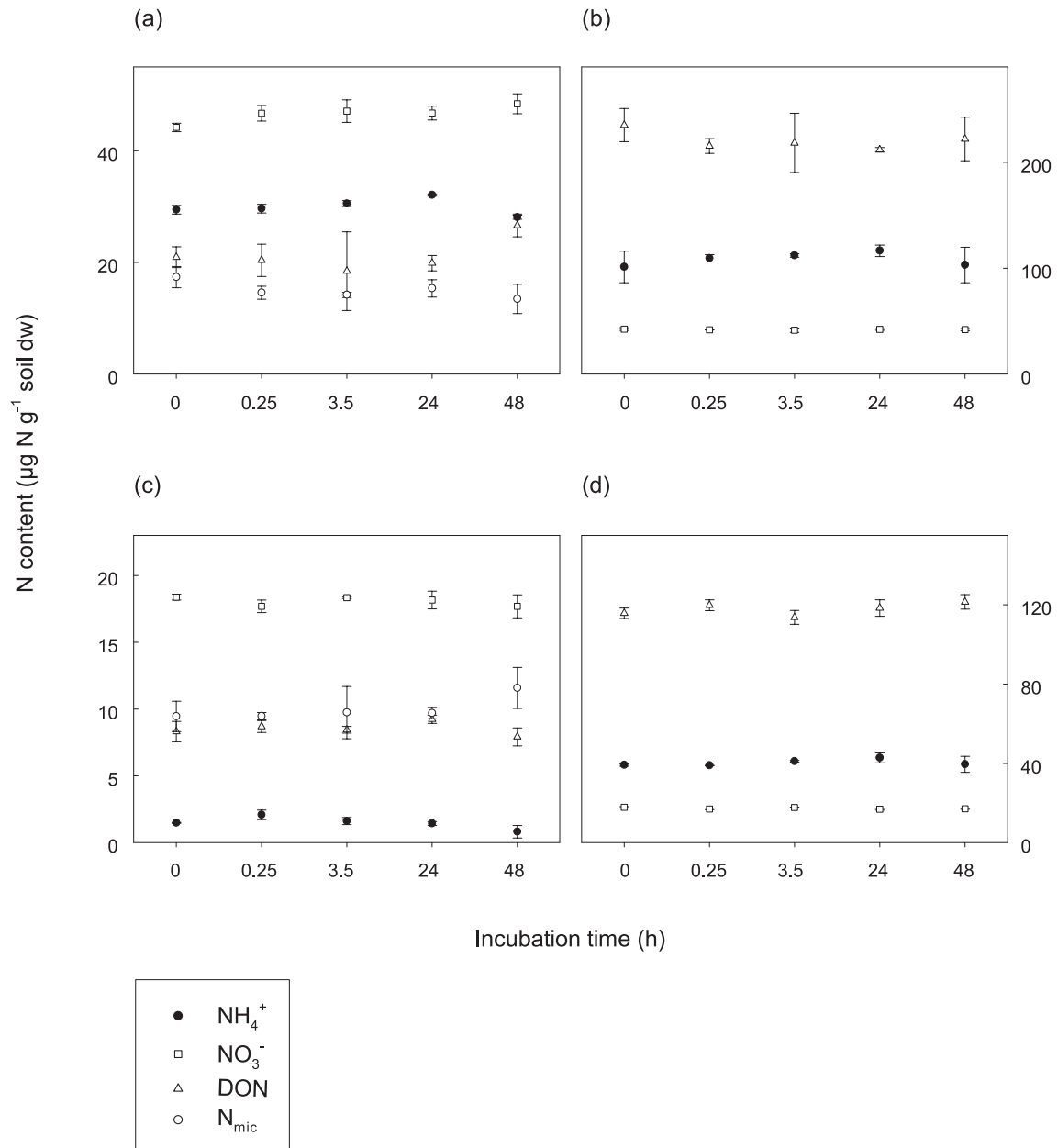


Figure 1.1.: Mean N content ($\mu\text{g N g}^{-1}$ soil dry weight, ± 1 SE) measured in different N pools (NH_4^+ , NO_3^- , DON, N_{mic}) over incubation time in the live forest soil (a), the sterile forest soil (b), the live grassland soil (c), and the sterile grassland soil (d) ($n = 3$)

Table 1.1.: Significance of the change in N content ($\mu\text{g N g}^{-1}$ soil dry weight) over incubation time in the different N pools measured in the forest soil and the grassland soil, live and sterile. Significance was tested using Pearson's product-moment correlation (r). Increase or decrease was considered significant when $P < 0.05$

Soil	N pool	live r	P	sterile r	P
Forest	NH_4^+	-0.28	0.3070	-0.06	0.8362
	NO_3^-	0.53	0.04342	0.01	0.9805
	DON	0.56	0.03013	0.10	0.7316
	N_{mic}	-0.37	0.1695	–	–
	N_{fixed}	-0.34	0.5180	–	–
Grassland	NH_4^+	-0.74	0.0016	0.13	0.6562
	NO_3^-	-0.28	0.3149	-0.43	0.1083
	DON	-0.21	0.4498	0.48	0.07185
	N_{mic}	0.57	0.02746	–	–
	N_{fixed}	0.03	0.9617	–	–

Table 1.2.: Significance of the change in atom % enrichment in ^{15}N (atom % ^{15}N) over incubation time in the different N pools measured in the forest soil and the grassland soil, live and sterile. Significance was tested using Pearson's product-moment correlation (r). Increase or decrease was considered significant when $P < 0.05$

Soil	N pool	live r	P	sterile r	P
Forest	NH_4^+	-0.95	< 0.0001	-0.32	0.2492
	NO_3^-	0.99	< 0.0001	-0.22	0.4378
	DON	0.48	0.0698	0.17	0.5341
	N_{mic}	-0.50	0.0584	–	–
	N_{fixed}	0.96	0.0022	–	–
Grassland	NH_4^+	-0.68	0.0057	-0.76	0.0009
	NO_3^-	0.81	0.0003	-0.19	0.5012
	DON	0.03	0.9231	0.09	0.7389
	N_{mic}	-0.533	0.0407	–	–
	N_{fixed}	1	< 0.0001	–	–

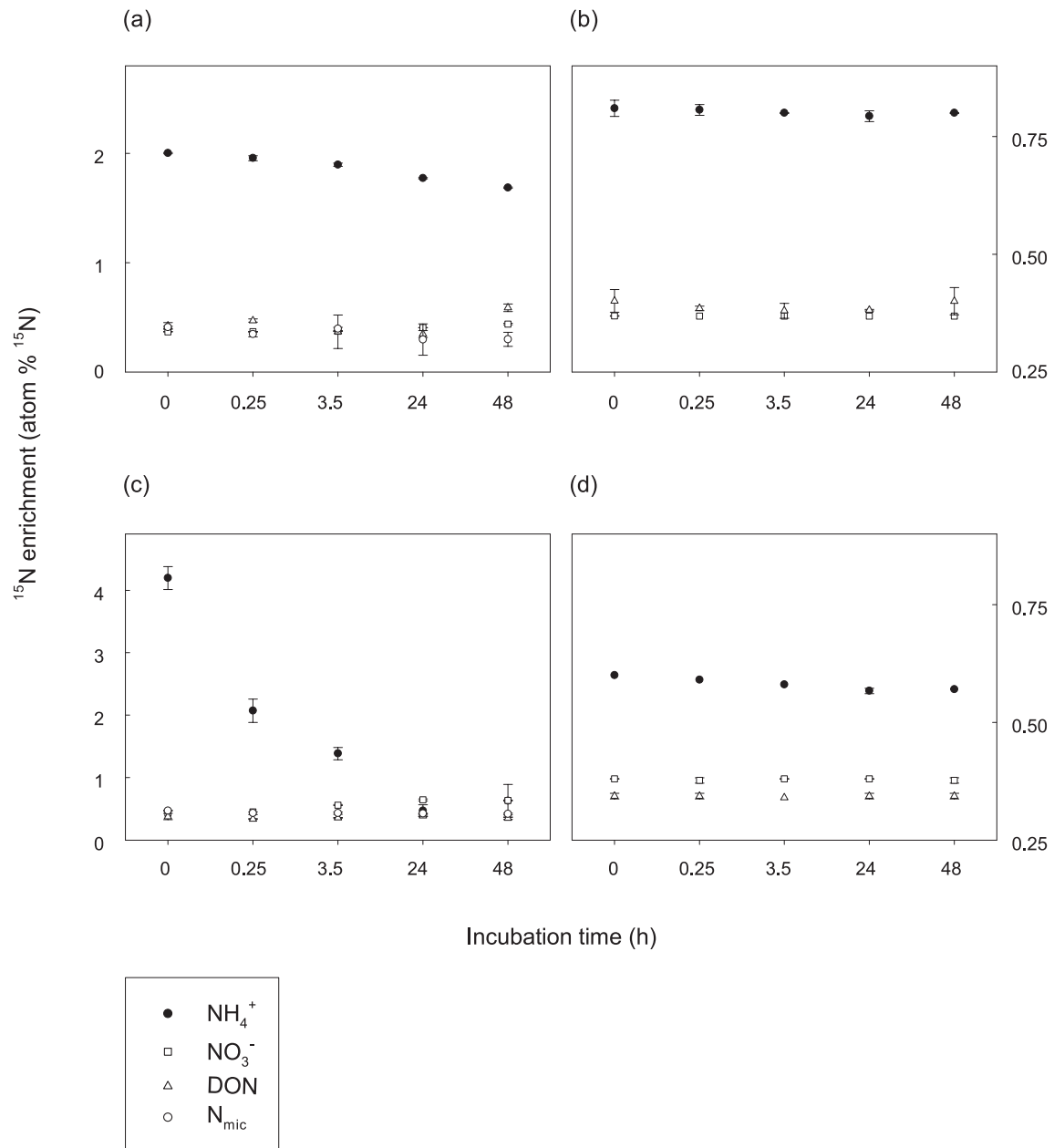


Figure 1.2.: Mean enrichment in ^{15}N (atom % ^{15}N , ± 1 SE) measured in different N pools (NH_4^+ , NO_3^- , DON, N_{mic}) over incubation time in the live forest soil (a), the sterile forest soil (b), the live grassland soil (c), and the sterile grassland soil (d) ($n = 3$)

Table 1.3.: Significance of the change in ^{15}N recovery (%) of the added $^{15}\text{N-NH}_4^+$ over incubation time in the different N pools measured in the forest soil and the grassland soil, live and sterile. Significance was tested using Pearson's product-moment correlation (r). Increase or decrease was considered significant when $P < 0.05$

Soil	N pool	live r	P	sterile r	P
Forest	NH_4^+	-0.94	< 0.0001	0.26	0.3458
	NO_3^-	0.96	< 0.0001	0.01	0.9697
	DON	0.66	0.0075	0.16	0.5626
	N_{mic}	-0.49	0.0647	–	–
	N_{fixed}	0.99	0.0002	–	–
Grassland	NH_4^+	-0.94	< 0.0001	-0.63	0.0122
	NO_3^-	0.91	< 0.0001	-0.08	0.777
	DON	0.00	0.9997	0.00	0.9992
	N_{mic}	-0.29	0.2944	–	–
	N_{fixed}	0.93	0.0071	–	–

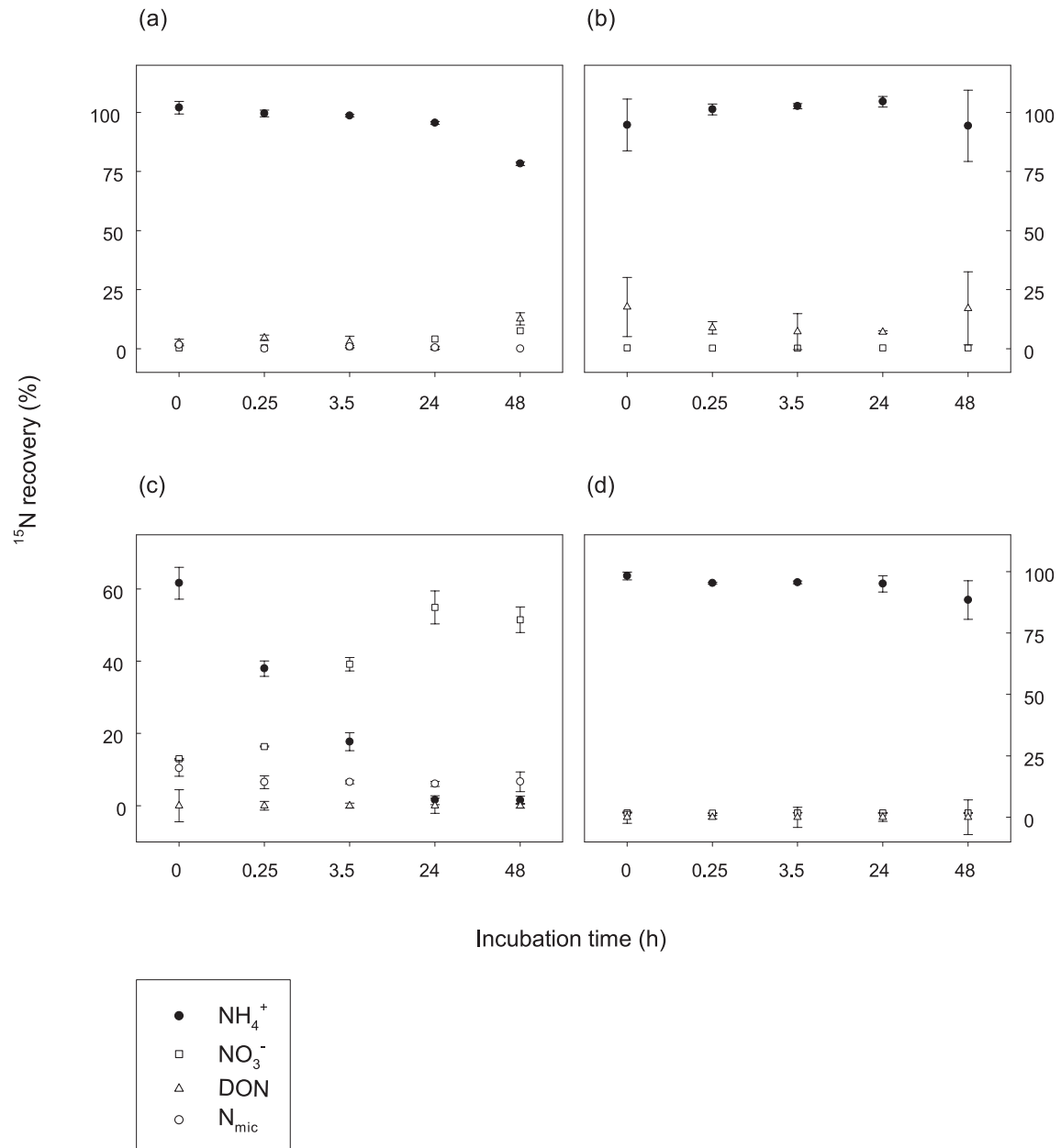


Figure 1.3.: Mean recovery rate of added ^{15}N ($\%, \pm 1 \text{ SE}$) measured in different N pools (NH_4^+ , NO_3^- , DON, N_{mic}) over incubation time in the live forest soil (a), the sterile forest soil (b), the live grassland soil (c), and the sterile grassland soil (d) ($n = 3$)

2. Zusammenfassung

Bei kurzfristigen Versuchen zur Bestimmung von Brutto-Umsatzraten von Stickstoff (N) in Boden mit der ^{15}N -Verdünnungsmethode, der sogenannten “isotope pool dilution technique”, wird häufig eine rasche Abnahme der ^{15}N Anreicherung im Ammonium-pool ($^{15}\text{N-NH}_4^+$) beobachtet. Unklar ist jedoch, welche Bodenprozesse für die sekundenschnelle $^{15}\text{N-NH}_4^+$ Aufnahme verantwortlich sind und ob es im Falle einer derart raschen Aufnahme des Isotopenindikators noch während der Inkubationszeit zu einem Rückfluss von ^{15}N in den Ammonium-pool kommen kann. Dies würde bei der Berechnung von Brutto-N-Mineralisierungsraten zu einer Unterschätzung der Rate führen. Das Ziel dieser Studie war daher zum einen zwischen biotischer und abiotischer Aufnahme von $^{15}\text{N-NH}_4^+$ zu unterscheiden sowie die Senken für $^{15}\text{N-NH}_4^+$ und den zeitlichen Verlauf der Aufnahmeprozesse über 48 Stunden genau zu untersuchen. Des Weiteren sollte geprüft werden ob es während einer Inkubationszeit von 48 Stunden zu einem Rückfluss des Isotopenindikators kommt und welchen Einfluss dieser Rückfluss auf die Brutto-N-Mineralisierungsraten haben könnte. Für den experimentellen Versuch wurden Bodenproben (Oberboden: 0–10 cm) aus einem Buchenwald und einem Grasland in Österreich verwendet, die sich in ihrer NH_4^+ -Konzentration und in der Aufnahmegeschwindigkeit von $^{15}\text{N-NH}_4^+$ unterschieden. Um eindeutig zwischen biotischen und abiotischen Bodenprozessen unterscheiden zu können, wurden diese teilweise mittels Autoklavieren sterilisiert. Die sterilen und nicht-sterilen Böden wurden anschließend mit $^{15}\text{N-NH}_4^+$ isotopisch markiert. Bei ansteigender Inkubationszeit (0, 0.25, 3.5, 24 und 48 Stunden), wurde jeweils die NH_4^+ -Konzentration

2. Zusammenfassung

sowie die ^{15}N Anreicherung in den inorganischen, organischen und mikrobiellen Stickstoff-pools gemessen um damit die biotische Aufnahme von Ammonium nachzuvollziehen. Des Weiteren wurde die abiotische Fixierung von NH_4^+ zum Zeitpunkt 0 und 24 Stunden nach ^{15}N Zugabe gemessen. Hier wurde außerdem zwischen der von Tonmineralien und der von Huminstoffen fixierten NH_4^+ -Fraktion unterschieden. Über die Gesamtdauer des Experiments betrug die Wiederfindungsrate des ^{15}N Isotopenindikators in beiden Böden mehr als 100 %. Im Graslandboden, der eine rasche $^{15}\text{N}\text{-NH}_4^+$ Abnahme aufwies, wurde das isotopisch markierte NH_4^+ nur wenige Sekunden nach Zugabe größtenteils abiotisch durch die Diffusion in Tonschichten fixiert (24 %). Zusätzlich zeigten sich mikrobielle Aufnahme (10 %) und Nitrifizierung (13 %) für die rasche Abnahme der $^{15}\text{N}\text{-NH}_4^+$ -Anreicherung verantwortlich. Sowohl für den Graslandboden mit rascher NH_4^+ -Umsetzung als auch für den Waldboden wurde auf Grund der mit der Inkubationszeit ansteigenden Wiederfindungsraten von ^{15}N ein Rückfluss des Isotopenindikators in den verfügbaren Ammonium-pool ausgeschlossen. Eine Sensitivitätsanalyse der Daten ergab, dass ein derartiger Rückfluss immensen Einfluss auf die Brutto-N- Mineralisierungsraten haben könnte (~60 % Unterschätzung der Rate im Graslandboden). In dieser Studie konnte gezeigt werden, dass sogar bei rascher biotischer Immobilisierung oder abiotischer Fixierung von $^{15}\text{N}\text{-NH}_4^+$ innerhalb von 24 Stunden Inkubationszeit und wahrscheinlich sogar bis zu 48 Stunden ein Rückfluss des Isotopenindikators sehr unwahrscheinlich ist. Demzufolge muss in kurzfristigen ^{15}N -Verdünnungsexperimenten auch im Falle rascher $^{15}\text{N}\text{-NH}_4^+$ Aufnahme bei der Berechnung von Brutto-N-Mineralisierungsraten der Rückfluss von ^{15}N in den Ammonium-pool nicht berücksichtigt werden.