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Use of natural abundance of $\delta^{15}N$ as indicator of long-term N management on grassland farms - An estimation of long-term N efficiency

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I. Abstract

Productive agroecosystems usually rely on the addition of Nitrogen (N) generally as chemical synthetic (synonymus with mineral) N fertilizers and animal excreta, but inefficient N management nitrogen losses contribute to environmental pollution, global warming, and a decline in biodiversity. This thesis was designed to test the hypothesis that the natural abundance of $\delta^{15}N$ signatures of various N pools may be used to reconstruct the efficiency of long-term N management of grassland farms retrospectively. For this I assumed that N losses should preferably go along with a loss of ^{14}N forms, i.e., left behind is nitrogen that is enriched in ^{15}N.

To test this hypothesis, two types of long-term fertilizer experiments in a low mountain range pasture ecosystem at the former Grassland Research Station in Rengen (Eifel Mountains) of the University Bonn, Germany, were considered to examine the changes in natural N isotope composition of various N pools. In a first experiment, a chemical synthetic fertilizer (calcium ammonium nitrate; $\delta^{15}N$ = -1.0 ‰) was applied at a rate of 0 to 240 kg N $ha^{-1}a^{-1}$ combined with a two and four cut management throughout 22 years. In a second study, a field lysimeter experiment, chemical synthetic fertilizer (calcium ammonium nitrate; $\delta^{15}N$ = -1.0 ‰) and cattle slurry ($\delta^{15}N$ = 8.9 ‰) were applied. N was applied at rates of 0 to 480 kg N $ha^{-1}a^{-1}$ for a time period of 22 years. Sampling different plant species and soils or particulate organic matter (>250 µm, 63-250 µm) then allowed to relate excess N fertilization and resulting N losses to the cumulative changes of $\delta^{15}N$ signatures. Based on these N isotope data the study was extended by a survey on nine common grassland farms, located in North Rhine-Westphalia and Rhineland-Palatinate in Germany and once in Styra, Austria. These farms comprised different N management, and could be grouped into low and high N input categories thus served as an independent set of test sites to verify the use of $\delta^{15}N$ signature as an indicator of practically relevant N management on grassland farms. Samples from these farms consisted of soil, plant biomass, hay, silage, milk, hair, urine, faeces, slurry, and manure.

The results showed that the mean $\delta^{15}N$ values of plant biomass (-1.3 to 0.95 ‰) were approximately 2-5 delta units lower compared with those of the topsoils (3.1 to 3.7 ‰), suggesting that light N was lost during soil organic matter formation. The variations in plant $\delta^{15}N$ values can be attributed to differences in plant growth, resource acquisition strategies, or $\delta^{15}N$ values of the soil N pools for which they compete. No significant differences were observed between low and and high N input

management, neither in the plant biomass nor in the soil samples. Obviously, chemical synthetic N fertilizers applied at moderate rates did not affect the ^{15}N signatures of plants biomass and soils significantly.

If higher N fertilizer rates were applied (up to 480 kg N ha^{-1}), the mean $\delta^{15}N$ increased from -1.2 ‰ to 4.8 ‰ for plants, and from 1.8 ‰ to 6.0 ‰ for the topsoils, respectively. The largest enrichment of ^{15}N was found in samples that were manured with organic fertilizer. As total soil N contents did not change significantly during the fertilization experiment, and since N losses via leaching were negligibly small, it was concluded that beside plant uptake and removal of N with biomass, most N was lost from the field plots through the gaseous phase. Particularly, N volatilization processes may account for a significant ^{15}N isotope enrichment. In soils, this enrichment of $\delta^{15}N$ values was preserved, suggesting that the respective N was incorporated into soil organic matter pools with slow N turnover.

The screening of different farm types confirmed a significant variation in the $\delta^{15}N$ values of harvested plant materials (-2.18 ‰ to 6.79 ‰) and the topsoils (1.47 ‰ to 7.91 ‰). Relative to farms under low N input management, the ^{15}N of samples taken from high N input managed grasslands were elevated by 2.8 delta units. Moreover, mean $\delta^{15}N$ values of plant biomass, soil and hair were closely correlated with stocking rate and milk production. High N input farms with a stocking rate larger than 1.4 livestock units/ha were characterized by higher amounts of organic N return per area and hence, higher losses of N from the slurry or farmyard manure, being reflected in elevated $\delta^{15}N$ values of the studied N pools. In turn, lower $\delta^{15}N$ values of topsoils and plants were related to low emission fertilizer application techniques and larger distances between stable and field.

Overall, my results support the hypothesis that high N input management on grassland farms systematically influences the $\delta^{15}N$ values of soils and plant biomass. Hence, the $\delta^{15}N$ signatures of different farm N pools, notably plant biomass, soil and hair could be identified as useful indicators for the efficiency of N management, i.e. the $\delta^{15}N$ tracing technique represents an attractive approach to reconstruct the retrospective intensity and efficiency of fertilizer management in agricultural practice.

II. Zusammenfassung

Produktive Agrarökosysteme sind auf den Einsatz von Stickstoff (N) angewiesen, welcher hauptsächlich als chemisch-synthetischer Dünger und in Form von tierischen Exkrementen dem Boden und darüber schließlich den Pflanzen zugeführt wird. Sie können jedoch – je nach Art und Höhe der Düngung – maßgeblich zur Umweltverschmutzung beitragen. Sie sind erheblich am Prozess der globalen Erwärmung beteiligt und führen zu einer verringerten Biodiversität.
Ziel dieser Arbeit war es, die Hypothese zu testen, dass sich das natürliche $\delta^{15}N$ Isotopenverhältnis (Isotopensignatur) von diversen N-Pools im landwirtschaftlichen Betrieb dazu eignet, Rückschlüsse auf den langfristigen Stickstoffeinsatz und dessen Effizienz in Grünlandbetrieben zu ziehen. Dafür nehme ich an, dass Stickstoffverluste mit dem Verlust des leichten ^{14}N Isotops einhergehen und folglich zu einer Anreicherung des schweren ^{15}N Isotops in dem zurückbleibenden N-Pool führen.

Zwei Langzeitexperimente an der Lehr- und Forschungsanstalt der Universität Bonn in Rengen (Eifel) sind hinsichtlich der Veränderungen der natürlichen Isotopenzusammensetzung von unterschiedlichen N Pools untersucht worden.
In dem ersten, 22-jährigen Experiment wurde ausschließlich chemisch-synthetischer Dünger (Kalkammonsalpeter; $\delta^{15}N$ = -1.0 ‰) in Raten von 0 bis 240 kg N $ha^{-1}a^{-1}$ verwendet und mit einer Zwei- und Vierschnittnutzung kombiniert. Bodenproben sowie ausgewählte Pflanzenarten wurden hinsichtlich ihrer $\delta^{15}N$ Signatur untersucht.
Im zweiten Experiment, einem 20-jährigen Lysimeterversuch, wurde sowohl chemisch-synthetischer Dünger (Kalkammonsalpeter; $\delta^{15}N$ = -1.0 ‰) als auch organischer Dünger (Rindergülle; $\delta^{15}N$ = 8.9 ‰) eingesetzt, welche kontinuierlich in Raten von 0 bis 480 kg N $ha^{-1}a^{-1}$ über einen 22-jährigen Zeitraum appliziert wurden. Durch die Analyse der pflanzliche Biomasse, Boden und unterschiedliche Fraktionen des Bodenmaterials (> 250 µm, 63 - 250 µm) ließen sich Rückschlüsse auf eine überschüssige Stickstoffdüngung und potentielle Stickstoffverluste ziehen, da diese zu einer kumulativen Veränderung der $\delta^{15}N$ Signatur führten.

Basierend auf den Ergebnissen dieser beiden Langzeitversuche an der Lehr- und Forschungsanstalt der Universität Bonn in Rengen (Eifel) sind die Untersuchungen auf neun herkömmlichen Grünlandbetrieben erweitert worden. Damit sollte die Frage beantwortet werden, ob sich die $\delta^{15}N$ Signatur grundsätzlich auch als Indikator für

den N-Einsatz in Praxisbetrieben eignet. Dafür sind acht Grünlandbetriebe in Deutschland (Nordrhein-Westfalen und Rheinland-Pfalz) und ein Betrieb in Österreich (Steiermark) untersucht worden. Die Betriebe unterschieden sich hinsichtlich ihrer Bewirtschaftungsintensität und wurden daher in die Kategorien niedrige (extensitiv) und hohe (intensiv) N Aufwandmenge klassifiziert. Von den Betrieben sind im Frühjahr/Sommer und Herbst 2007 aus verschiedenen N Pools (pflanzliche Biomasse, Boden, konserviertes Futter, Exkremente, Gülle, Milch, Haare) Proben entnommen und auf ihre $\delta^{15}N$ Signatur analysiert worden.

Die Ergebnisse des ersten Langzeitversuches zeigten, dass die durchschnittlichen $\delta^{15}N$ Werte der pflanzlichen Biomasse (-1.3 bis 0.95 ‰) um etwa 2 - 5 ‰ weniger stark mit ^{15}N angereichert sind verglichen mit den durchschnittlichen $\delta^{15}N$ Werte der Oberböden (3.1 bis 3.7 ‰), was auf mögliche Verluste des leichten ^{14}N Isotopes während der Umsetzung der organische Bodensubstanz hinweist. Diese Schwankungen können durch Unterschiede im Pflanzenwachstum und den Mechanismen der Nährstoffaufnahme erklärt werden aber auch auf die verschiedenen $\delta^{15}N$ Werte der für sie verfügbaren N Ressourcen in der oberen Bodenschicht zurückgehen. Weder in der pflanzlichen Biomasse noch in den Bodenproben wurden signifikante Unterschiede zwischen den niedrigen und hohen N Düngungsvarianten festgestellt, obwohl Unterschiede zwischen Pflanzenarten gemessen wurden. Daher kann angenommen werden, dass chemisch-synthetische Dünger in betriebsüblicher Aufwandmenge die ^{15}N Signatur nicht signifikant beeinflusst.

Die auf bis zu 480 kg gesteigerte N Düngungsmenge führte zu erhöhten $\delta^{15}N$ Werten in der pflanzlichen Biomasse (von -1.2 ‰ bis 4.8 ‰) und in der oberen Bodenschicht (von 1.8 ‰ bis 6.0 ‰). Die größte Anreicherung an ^{15}N wurde in den Proben der organisch gedüngten Parzellen ermittelt. Da sich der Anteil des Stickstoffs im Gesamtboden während des Düngungsexperimentes nicht signifikant veränderte und die N Auswaschungsverluste vernachlässigbar gering waren, kann aus dieser Studie geschlussfolgert werden, dass neben der Stickstoffbindung in der pflanzliche Biomasse und des Stickstoffentzuges durch die Abfuhr des Erntegutes ein Großteil des Stickstoffs im gasförmigen Aggregatszustand verlorengegangen ist. Im Gegensatz zur Stickstoffaufnahme durch Pflanzen, führt insbesondere der Prozeß der Volatilisation zu signifikanten höheren $\delta^{15}N$ Werten. Die $\delta^{15}N$ Werte belegen, dass die Anreicherung des schweren ^{15}N Isotopes im Boden erhalten blieb. Dies lässt

darauf schließen, dass der Stickstoff in N Pools der organischen Bodensubstanz eingebaut wurde, welche einer langsamen N Umsetzung unterliegen.

Bei der Untersuchung der N Pools unterschiedlicher Höfe konnten ebenfalls signifikante Unterschiede der $\delta^{15}N$ Werte in der pflanzlichen Biomasse (-2.18 ‰ bis 6.79 ‰) und der oberen Bodenschicht (1.47 ‰ bis 7.91 ‰) beobachtet werden. Die $\delta^{15}N$ Signaturen der Proben aus intensiv wirtschaftenden Grünlandbetrieben waren im Vergleich zu den Proben aus extensiv bewirtschafteten Grünlandbetrieben um 2.8 ‰ erhöht. Ein weiterer Hinweis auf die Wirkung der verlustreichen organischen Düngung ergab sich aus den gemittelten $\delta^{15}N$ Werten der pflanzlichen Biomasse, des Oberbodens und der Haare; sie waren stark positiv korreliert mit der Besatzstärke und der Milchproduktion. Intensiv wirtschaftende Betriebe mit einer Besatzstärke von 1.4 GVE/ha sind charakterisiert durch einen höheren Anfall an organischem Stickstoff pro Flächeneinheit. Dies resultiert in Stickstoffverlusten in der Gülle oder des Festmistes, welche durch erhöhte $\delta^{15}N$ Werte eindeutig angezeigt wurden. Daher stellen die $\delta^{15}N$ Signaturen von unterschiedlichen N Pools, insbesondere von pflanzlicher Biomasse, Boden und Haaren einen geeigneten Indikator für die Effizienz des Stickstoffmanagements dar. Weiterhin korrelieren niedrige $\delta^{15}N$ Werte des Oberbodens und der pflanzlichen Biomasse mit emmisionsarmen Applikationstechniken und höheren Distanzen zwischen dem Betriebsgelände und den Grünlandflächen.

Die Ergebnisse dieser Dissertation demonstrieren, dass die $\delta^{15}N$ Werte von Böden und pflanzlicher Biomasse durch N intensive Bewirtschaftung von Grünland signifikant beeinflusst werden. Daher stellen $\delta^{15}N$ Untersuchungen, insbesondere von Böden, pflanzlicher Biomasse und Haaren eine vielversprechende Möglichkeit zur rückwirkenden Einschätzung der Intensität und Beurteilung der Effizienz des Düngemanagements in der landwirtschaftlichen Praxis dar.

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VI. List of Acronyms

ANOVA	Analysis of variance
$\delta^{15}N$	Variation in stable nitrogen isotope ratios
$\Delta^{15}N$	Difference between measured and calculated $\delta^{15}N$
%	Percent
‰	Per mille
a	Year
atm.	Atmospheric
C	Carbon
CDT	Canyon Diablo meteorite
DM	Dry matter
GVE	Großvieheinheit
H	Hydrogen
kg	Kilogram
l	Liter
LU	Lifestock unit
manN	Manure N
minN	Mineral N
m.a.s.l.	Meters above sea level
mm	Millimeter
N	Nitrogen
N_2	Dinitrogen
N_2O	Nitrous oxide
NH_3	Ammonia
NH_4^+	Ammonium
NO	Nitric oxide
NO_3^-	Nitrate
O	Oxygen
R^2	Coefficient of determination
S	Sulfur
SOM	Soil organic matter
t	Tons
V-PDB	Vienna PeeDee formation
V-SMOW	Vienna mean standard ocean water

1 Introduction

1.1 Nitrogen in Agriculture

Besides Phosphorous and Potassium, Nitrogen (also referred to as N) is the most essential nutrient for crop growth. N is a necessary element of various genetic, metabolic and structural composites in plant cells. It is crucial for the formation of nucleid acids and proteins and for many other important biochemical processes. For example, it is contained in clorophyll molecules that are necessary to perform photosynthesis (Keeney and Hatfield, 2008). Hence, limitation of the macronutrient nitrogen leads to diminishing crop growth, and as a consequence decreasing yields. Nitrogen fertilization is applied worldwide since supplementary N application is required to increase the productivity within the most agroecosystems. It accounts for approximately 40 % of the increase in food production during the past 50 years (Mosier et al., 2001). The major sources of N applied in agriculture are biological N_2-fixation through plants and microorganism, organic manure and chemical N_2 fixation via industrial processes.

The increase of world population resulting in higher demands of food, forage crops and fuel production significantly increases the fertilizer consumption as shown in Table 1 (Galloway, 1998; FAO, 2008).

Table 1: World fertilizer supply and demand, 2007/2008 – 2011/2012 (modified after FAO, 2008)

	2007/2008	**2008/2009**	**2009/2010**	**2010/2011**	**2011/2012**
		(thousand tonnes)[1]			
Total supply	206 431	212 225	219 930	230 334	240 711
Total demand	197 004	201 482	205947	211 230	216 019
Surplus	9 427	10 743	13983	19 104	24692

[1] Difference between supply and potential consumption.

In the more industrialized countries a shift in food consumption is observed towards higher proportions of meat and at the same time, animal husbandry becomes more and more industrialized. This trend is accompanied by an increasing number of animals per herd leading to large amounts of organic manure produced on intensive farms. Specifically for livestock farms, the amount of accumulated and applied

excreta often exceeds the nutrient demands of plants. As a consequence, N recycled in manure is not applied at adequate rates. Animals that were raised traditionally on grassland are nowadays increasingly kept indoor all year round. This leads to an accumulation of large amounts of manure that cannot be redistributed back to the areas where arable crops were originally grown, since the purchased feed was produced off-farm (Koelsch, 2005). The feeding diets are higher in crude proteins based on small grain cereals, maize and soybeans, which in turn alter the nitrogen content of animal manure (Paul et al., 1997). In several countries in the EU, such as UK and the Netherlands, dairy farms are a significant source of N pollution (Castillo et al., 2000). Thus, higher N efficiency is requested to reduce the amout of fertilizers needed for high crop yields and to minimize environmental problems caused by excessive N usage.

1.2 Environmental problems caused by high fertilizer N application

Besides the benefits in yields associated with nitrogen fertilizer application, the surplus application has detrimental impacts on the environment. Excessive N fertilization results not only in unwanted changes of the soil nutrient status, but is also responsible for potential losses via various pathways (Domburg et al., 2000; Van Beek et al., 2003).

Nitrogen is a reactive and mobile element. Hence, chemical conversions such as nitrification, denitrification, nitrous oxide formation, leaching of nitrate and volatilization of ammonia may cause unwanted side effects (Dittert et al., 1998; Townsend et al., 2003). High doses of N in the atmosphere fertilize adjacent ecosystems, and thus N leakage may occur, which can even result in a lowered biodiversity (Oenema et al., 2001; Canfield et al., 2010). Moreover, nitrous oxide and ammonia are greenhouse gases that contribute to the stratospheric ozone destruction and global warming (Lashof and Ahuja, 1990; Canfield et al., 2010). Furthermore, excess of N in lakes, coastal waters and estuaries stimulate an overproduction of biomass and the subsequent decay under anearobic conditions. Therefore, it is commonly regarded as main reason for hypoxia (Keeney and Hatfield, 2008).

1.3 Improving and monitoring N efficiency

Nutrient budgets of ecosystems were introduced to better understand the nitrogen pathways within the nutrient cycles. They are used as indicators for nutrient management (Oenema, 2003). Long-term N imbalances are contradictory to sustainable agriculture. Hence, the use of nutrient budgets as indicators and policy instruments for nutrient management practices was proposed (Oenema, 2004). Direct regulations by the public surveillance were introduced advising farmers to reduce environmental damage caused by surplus of nitrogen fertilizers. Economic and political pressure, such as prices for nitrogen fertilizers, high cost of energy, nutrient taxes (Netherlands), fines (Germany) for unefficient animal waste applications or the legal regulated area with green crops in winter to reduce nutrient leaching and run-off (Denmark), help to avoid N imbalances (Oenema et al., 2003; Ondersteijn et al., 2003). Additionaly, management practices such as limitating the livestock unit (LU) per ha, the use of nitrification inhibitors and precision farming or plant breeding are beneficial in this respect.

Thus, reliable and easily applicable indicators are also requested by farmers to improve their technical skills (Breembroek et al., 1996; Brouwer, 1998; van de Molen et al., 1998; Halberg et al., 2005). A wide range of examinations were carried out addressing e.g. nutrient budgets or balances (Oenema et al., 2003), input-output accounting systems (Goodlass et al., 2003) and farm-gate budgets (Watson et al., 1999; Öborn et al., 2003; Haas et al., 2007). The balances were created at different scales, ranging from the farm system (Bassanino et al., 2007) to regional (Sacco et al., 2003) and to global scales (Van der Hoek et al., 1999).

Within farm systems, several types of nitrogen budgeting are in common use, such as (i) element or farm-gate budgets (Öborn et al., 2003), (ii) soil-surface budgets (SSB) (Bassanino et al., 2007), and (iii) soil-system budget whose accuracy increases in the same order (Oenema et al., 2003).

Futhermore, balances are used as a regulatory policy instrument since farm-gate budgets are published in annual farm statistics. Hence, budgeting approaches at farm-scale that compare nutrient inputs and outputs are frequently used to estimate N efficiency (Jarvis, 1999; Domburg, 2000; Cassman et al., 2002). Oenema et al. (2003) have described nutrient budgets as a sum of nutrient inputs and outputs from a defined agricultural farming system over a fixed time period. Their budgets include N inputs such as mineral N fertilizers, animal excreta, biological N fixation, atmospheric nitrogen deposition and N outputs by removing crops or fodder. Budgets

based on the farm-gate balance also include N outputs through the produced milk or meat. The difference between N input and output is used as an indicator for either nutrient surplus or deficit. As a consequence, each import and export of nutrients has to be registered and balanced in more advanced farming practise.

In contrast to the farm-gate balances that consider N inputs and losses for the farm without specifiying the spatial distribution, other budget systems such as the soil surface or the soil-system budget are more appropriate to recover N losses for small scale systems. While the soil-surface budget records all nutrients that enter and leave the soil but still excludes N losses through volatilization (OECD, 2001), the soil-system budget includes all pathways that lead to a net nutrient reduction or accumulation within the system in question.

However, N losses arising from e.g. the stable or from manure storage systems are not taken into account in any of these three budgets (Oenema et al., 2003). Moreover, each of the three methods is complicated by animal husbandry which in grassland is practically essential. In addition, N_2-fixation by legumes may play a major role in grassland farming (Jarvis, 1993).

Nitrogen surplus indicates potential N losses, but without considering the various environmental interactions that control N sinks and sources at different farm hot-spots (Bacon et al., 1990). However, respective measurements on farmland and in laboratory are missing in most cases. For a well-balanced N input managment, farming systems with reduced N input specifically benefit from governmental assistance, however, N imbalances are still difficult to discover (Oenema, 2003), particularly if spatial distribution of nitrogen input within the farm strongly varies (Bacon et al., 1990; Van Beek et al., 2003). Moreover, due to considerable amounts of estimated N losses, which are not easely assessable, nutrient balances are rarely precise. Thus, it would be of great advantage to have a reliable and simple indicator for N efficiency in order to trace back nutrient imbalances, specifically after prolonged high or low N input management. Such an indicator of the N input-output balances at a given field plot or farm may be derived from $\delta^{15}N$ natural abundance measurements.

1.4 Stable Isotopes

Atoms consist of positively charged protons, negatively charged electrons and uncharged neutrons. Both protons and neutron have masses that are approximately equal, whereas the mass of an electron is three orders of magnitude smaller. A

chemical element is determined by the number of protons in its nucleus. The number of protons equals the number of electrons, located in a negativly charged cloud around the nucleus. The proton number of a given chemical element is always the same, whereas the number of neutrons can vary. This leads to differences in the atomic mass of the respective chemical element. Elements with the same number of protons but with variations of the atom weight resulting from a different number of neutrons in the nucleus are called isotopes (Rundel et al., 1988; Chriss, 1999; Dawson and Brooks, 2001; Sharp, 2006; Fry, 2008). Isotopes (greek *isos* = equal, *topos* = place) can exist in both stable and unstable (radiactive) forms. In contrast to radioactive isotopes, stable isotopes do not appear to decay to other isotopes. Stable isotopes are widely distributed in organic and inorganic compounds found in the soil, atmosphere, and all living organisms. The analysis and detection of stable isotopes are based on their different atomic masses. Most elements of ecological and biological interest, such as carbon (C), nitrogen (N), sulfur (S), hydrogen (H) and oxygen (O) have two or more stable isotopes. Typically one of them occurs to be dominant, while the others are usually rare (see Table 2), which offers the possibility to use the distribution of a certain element specific stable isotope as an indicator for various chemical and biological processes.

Table 2: Summary of the natural abundance of stable isotopes for chemical elements of great interest in ecological and environmental studies and their reference standard (Fry, 2006).

Element	Isotope	Abundance (%)	Ratio measured	Standard
H	^{1}H	99.94	$^{2}H/^{1}H$	V-SMOW[a]
	^{2}H	0.016		
C	^{12}C	98.89	$^{13}C/^{12}C$	V-PDB[b]
	^{13}C	1.11		
N	^{14}N	99.64	$^{15}N/^{14}N$	N_2-atm.[c]
	^{15}N	0.36		
O	^{16}O	99.76	$^{18}O/^{16}O$	V-SMOW
	^{18}O	0.20		
S	^{32}S	95.02	$^{34}S/^{32}S$	CDT[d]
	^{34}S	4.21		

[a] The original standard was standard mean ocean water (SMOW) which is no longer available. Vienna-SMOW is available from the IAEA.
[b] The original standard was a belemnite from the PeeDee formation (PDB) which is no longer available, Vienna-PDB is available from the IAEA
[c] atm. = atmospheric
[d] Canyon Diablo meteorite

In contrary to the chemical properties of an atom or molecule, which are determined by the configuration of the respective electrons, varying atom masses of isotopes are the reason for small differences in physical properties. Variations of the abundance of stable isotopes in a chemical compound are based on the isotope effect or so-called fractionation. Enzymatic discrimination and differences in kinetic characteristics and equilibria can result in reaction products that are isotopically heavier (enriched) or lighter (depleted) than their precursor materials (Lajtha and Michener, 1994). The rate at which an isotopic species passes through a thermodynamically driven reaction is partly controlled by the energy required to dissociate the molecule (Urey, 1947). Light isotope bonds have more potential energy than heavy isotope bonds since the cleavage of light isotope bonds requires less energy (Bigeleisen, 1965). As a consequence, the light isotope species usually reacts faster during a chemical reaction.

In general, the isotope ratio (R) is defined as follows (Fry, 2006):

$$R = \left(\frac{X_h}{X_l} \right) \quad , \qquad (1)$$

where X_h refers to the heavy and X_l to the light isotope. The stable isotope composition (or synonymously the natural isotope abundance) is usually expressed in delta (δ) units, where the isotope ratio R of a sample is related to that of an international standard (see Table 2). Since the mass differences are very small compared to the absolute masses, the natural abundances are expressed in parts per thousands (or per mille) [‰] (Mariotti, 1984):

$$\delta R[‰] = \left[\left(\frac{R_{sample}}{R_{standard}} \right) - 1 \right] \cdot 1000 \quad . \qquad (2)$$

A compound is considered as enriched relative to the international standard, if it shows a higher δ value in its isotope signature. In contrast, if the compound shows a lower δ value, it denotes a depletion of the heavier isotope and an enrichment of the lighter one relative to the international standard.

The extent of isotope fractionation during a chemical reaction is quantitatively described by the fractionation factor α (Sharp, 2006):

$$\alpha = \frac{R_A}{R_B} \quad , \qquad (3)$$

where R_A is the isotope ratio of the participating compound A, and R_B that of the corresponding compound B.

Obviously, processes without isotope fractionation have a fractionation factor of α = 1. Since the α values are typically close to 1, the fractionation factor is usually converted to the isotope enrichment factor ε given in per mille [‰] (Högberg, 1997):

$$\varepsilon[‰] = (\alpha - 1) \cdot 1000 \quad . \qquad (4)$$

Isotope effects can only be observed if the reaction is incomplete. When all N atoms of the substrate molecule go into the product of the given reaction, the $\delta^{15}N$ values of the product is identical with that of the former substrate (Mariotti et al., 1981). Hence, in an unidirectional reaction, for which the substrate is not limited, the fractionation factor is constant. The fractionation factors observed for the various reactions within the nitrogen cycle range from –0.98 to 1.033 ‰ (Table 3).

Table 3: Fractionation factors for various processes in the N cycle (modified after Högberg, 1997).

Process	Fractionation factor α
N mineralisation	1.000
NH_3 volatilisation	1.029
Diffusion of NH_4^+ , NH_3 , NO_3^- in solution	~ 1.000
Nitrification	1.015 – 1.035
Denitrification	1.000 – 1.033
N assimilation	1.000 – 1. 002
N_2 fixation	0.980 – 1. 002
Metabolic steps in plants	0.980 – 1.020

The most significant $\delta^{15}N$ fractionations ocur for reactions resulting in N products that are emitted to the envrionment, such as denitrification, nitrifcation and volatilization. As a consequence, the remaining N-sources are enriched in ^{15}N (Högberg 1997).
The most common technique to measure isotope compositions is the isotope ratio mass spectrometry. The probing material is vaporised and the relative deviation of

the isotope ratio of the gaseous species is measured in comparison to a suitable standard (see Table 2).

1.5 Objectives of the study

The aim of this study was to test the hypothesis that measured $\delta^{15}N$ values of various N pools indicate the N efficiency after long-term N fertilization.

I assume that increased N fertilizer application is responsible for larger nitrogen losses resulting in critical N emissions to the environment. Biochemical processes that lead to N losses such as denitrification, nitrification and volatilzation are associated with high fractionation factors. Thus, products emitted to the environment are depleted in ^{15}N, whereas the remaining substrate must consequently be enriched in ^{15}N. Hence, increased $\delta^{15}N$ values of N pools in a given agroecosystem (soils, plants, animals) will be positively related to N losses.

The first part of the investigations was carried out on two long-term field trials on grassland that has been kept under controlled management for 20 years and 22 years. In the second part of the investigations, the central hypothesis was falsified on representative grassland farms. It was assumed that the $\delta^{15}N$ isotopic signature, which in turn is influenced by the N application technique and the distance between farmyard and pastures, provides insight into the N management practiced over the years retrospectively.

Experiment 1 (Chapter 2):

Do the $\delta^{15}N$ values of soil and plant biomass reflect the mineral N fertilizer management?

The aim of this primary investigation was to find out, whether $\delta^{15}N$ values of soil and plant biomass are suitable indicators of the underlying grassland management. All samples were derived from a grassland trial established 20 years ago at the Rengen Grassland Research Station of the University of Bonn. The experiment comprised two treatments with differing cutting frequency and N application rates characterizing low and high N input grassland managing systems.

I hypothesized that higher application rates of mineral N fertilizers and the resulting N losses may lead to increased $\delta^{15}N$ values. Hence, there should be significant differences in the $\delta^{15}N$ values between the low N input two´cut treatment and the high N input four cut treatment.

Experiment 2 (Chapter 3):

Is it possible to indicate the N efficiency after long-term fertilization (mineral and organic) by analysing the δ^{15}N values of soil and biomass?

In the second experiment the above-mentioned research question was tested on a long-term lysimeter experiment located at the University Bonn Grassland Research Station, which has been kept under the same N treatments for more than 20 years. Compared to the primary investigation (Experiment 1), both mineral and organic fertilizers were used at increased fertilizer application rates. The grassland plots were cut consistently four times per year. Samples of soil, leachates, roots and specific plant species, derived from the identical location with constant N- management were analysed. The objective of this experiment was to test whether observed δ^{15}N values within various N pools may be used as an indicator of N efficiency after long-term fertilization, i.e. monitoring the N losses that lead to critical N emissions to the environment according to input-output balances. It was also examined whether the δ^{15}N values of harvested plant biomass is indirectly influenced by floristic composition since specific isotope discrimination may differ among the plant species.

Experiment 3 (Chapter 4):

Is it possible to reveal the underlying N management on grassland farms using the natural abundance of ^{15}N of various N pools?

This experiment combines the results of the preceding two experiments. It was performed to test, whether it is possible to relate the results to common grassland farms practise. Hence, eight grassland farms in Germany and one in Austria were grouped into low and high N input categories.

The objective of this study was to establish a relationship between isotope ratios of several N pools of grassland farms and the underlying N management. I hypothesized that the isotopic signature of the N pools retrospectively reflects N efficiency, which in turn is influenced by the N application technique and the distance between farmyard and selected grassland plots. To this end, various N pools such as soils, feed components, fertilizers, and cattle tissues like milk, hair, faeces and urine were analysed.

2 Do the $\delta^{15}N$ values of soil and biomass reflect the mineral N fertilizer management?

2.1 Introduction

According to the UN Ecosystem Millenium Assessment, global N emissions have increased by a factor of three and continue to rise, with negative feedback mechanisms on biodiversity, environmental quality and human health (http://www.millenniumassessment.org). In agricultural systems, the nitrogenous fertilizers are needed to stimulate biomass production and to increase the protein content in plant material. However, high fertilizer rates also increase the risk of unwanted N losses by ammonia volatilization, leaching or microbial trace gas emission (Dittert et al., 1998). Higher N use efficiency is urgently required for both economic and ecological reasons.

Monitoring N use efficiency in agro-ecosystems may be achieved using sophisticated simulation models, such as DAISY, HERMES or EXPERT-N (Stenger et al., 1999; Kersebaum 2007). All these models, however, require numerous input variables, calibration and validation. It would be helpful, if the N balance of an agro-ecosystem could be more rapidly assessed by simple screening tools. Among other chemical attributes - the lighter ^{14}N isotope reacts more rapidly than the heavier ^{15}N due to lower bond strength, and hence accumulates in the products, whilst the residual (source) becomes enriched in ^{15}N (Mariotti et al., 1981; Wang et al. 2005). Here, the investigation of stable isotopes of N opens up new perspectives, as it provides insight into turnover and transfer processes within the soil N cycle. In general, all N transformations in any ecosystem lead to N isotope fractionation. Such $\delta^{15}N$ fractionations range from 0 ‰ to 35 ‰ for N_2 fixation and NH_3 volatilization, respectively (Högberg 1997). According to the Rayleigh model, (Robinson 2001) all N products emitted to the environment (NH_3, N_2O, NO, N_2, NO_3^-) are generally ^{15}N depleted. Hence, the remaining natural N-sources are enriched in ^{15}N (Högberg 1997). Compared to that, the degree of natural ^{15}N discrimination is low during mineralization or N uptake by the plants (Handley and Raven 1992). Therefore we hypothesize that $\delta^{15}N$ values in a given agroecosystem will be positively related to N

losses. In other words, the $\delta^{15}N$ signature is a potential but only rarely explored indicator for N use efficiency in agricultural sites (e.g. Watzka et al., 2006).
The aim of this study was, therefore, to elucidate whether $\delta^{15}N$ signatures of soil and plant biomass may also be used to reconstruct the intensity of nitrogen use in.a field-site experiment on plot scale. I hypothesized that high N input managed grassland farming and different intensive cutting systems could be traced back by increased $\delta^{15}N$ values of various samples.

2.2 Materials and Methods

2.2.1 Site description and experimental layout

The experimental site is located at the former Rengen Grassland Research Station of the University Bonn, in the Eifel Mountains, Germany (50°13´N, 6° 51´E) at 475 to 490 m.a.s.l. Long-term average annual precipitation was 811 mm, and the average annual temperature was 6.9°C (Schellberg et al., 1999). The topsoils at the sites were mainly Stagnic Cambisols with wet conditions after rainfall in winter, especially in the turf layer.

The experiment was established on former high N input managed pastures (Lolio-Cynosuretum), with two cuts and two grazing cycles per year. Until 1987 the field was fertilized in general with 200 kg N as mineral fertilizer and 15 m^3 cattle slurry per ha per year. The long-term trial was founded 1987 and was kept under the same conditions for 20 years. The plots were carried out in a randomised complete block design, with four blocks consisting of six different treatments.

The size of the individual plots was 9 x 3 m^2. The aim of the experiment was to compare two different grassland systems. Block A was held under high N input grassland management with four cuts at about the same time in May, July, September and October, whereas the plots in Block B were cut two times per year. All plots were fertilized with calcium ammonium nitrate containing 27.5 % of N. Total annual fertilizer rates ranged from 120 to 240 kg N ha^{-1} a^{-1} in the high N input managed block and from 60 to 120 kg N ha^{-1} a^{-1} in the low N input variety. In parallel, an unfertilized plot served as control in both blocks. Split doses of the fertilizers were applied immediately after every cut as described in Table 4.

Table 4: Details of the 20 years old long-term de-intensification experiment setting indicating the type and amounts of applied nitrogen (N) and number of cuts per year.

Grassland management	Treatment abbreviation	Applied Nitrogen[a] [kg $ha^{-1}a^{-1}$]	No. of cuts a^{-1}
Low N input	0, control	0/0	2
	minN 60	0/60	
	minN 120	40/80	
High N input	0, control	0/0/0/0	4
	minN 20	40/40/40/0	
	minN 240	60/60/60/60	

[a] calcium ammonium nitrate (27 % N)

2.2.2 Sampling and analyses of plant, soil and fertilizer

Bulk soil samples (n= 3) from at least ten randomized replicates were taken and pooled within each plot at 0-5 cm, 5-10 cm and 10-30 cm soil depth twice each in June and September 2006. Soil samples were sieved to < 2 mm and dried for 48 h at 100 °C.

Separate plant samples (n= 3) were taken from five species, *Lolium perenne* L., *Festuca rubra* L., *Taraxacum officinale* Web. and a mixed plant sample from *Trifolium repens* L. / *Trifolium pratense* L. Both species of clover (*Trifolium repens* L. / *Trifolium pratense* L.) were analyzed in a preliminary test separately. Plant material of the legume species was pooled in one sample, because their isotopic signature was similar but different from the non-legumes. Additionally, one sample of the total aboveground plant biomass was taken. To avoid edge effects among the plots, all biomass was clipped from the centre of the plots. The pooled samples were cut with a scissor 1 cm above top of mineral soil. All plant material was oven dried for 48 h at 60 °C. Samples of both plant and soil material were ground in a ball mill (Retsch, Germany) to achieve homogenous samples prior to ^{15}N analysis.

2.2.3 ^{15}N analysis

All ^{15}N analyses were conducted at the Technical University Munich, Chair of Grassland Science. Isotope ratios of $^{15}N/^{14}N$ and contents of total N in all samples

were determined by dry combustion in an elemental analyser (NA 1110; Carlo Erba, Milan, Italy) interfaced with an isotope ratio mass spectrometer (Delta Plus, Finnigan, Bremen, Germany). The isotopic ratios are expressed as $\delta^{15}N$ relative to ^{15}N air standards (Mariotti, 1984):

$$\delta R[‰] = \left[\left(\frac{R_{sample}}{R_{standard}} \right) - 1 \right] \cdot 1000 \quad , \qquad (5)$$

where R is the ratio of the abundance of $^{15}N/^{14}N$ of sample and standard. All samples were measured against laboratory standard gases, which were previously calibrated against the international standard (N_2 in the air) by use of the reference substances N-1 and N-2 for nitrogen isotope ratios provided by the IAEA, Vienna. The analytical precision was 0.2 ‰ for $\delta^{15}N$.

2.2.4 Statistics

Statistical analysis was performed separately for each experiment using SPSS 20.0 software (SPSS, Chicago, US). Differences between fertilizer treatments were analyzed by one-way analysis of variance (ANOVA) followed by a multiple range test (Scheffé post hoc test). If homogeneity of variance was missing, Dunnet´s T3 was calculated. For each sample, the 95 % confidence intervals on the mean were computed based on the sample mean and sample standard deviation.

2.3 Results

2.3.1 $\delta^{15}N$ in plants

Mean values of $\delta^{15}N$ of the above ground total plant biomass (Figure 1 and Figure 2) ranged from -0.7 ± 1.7 ‰ to 0.7 ± 0.4 ‰ for the low N-management and -1.3 ± 1.6 ‰ to 1.0 ± 1.4 ‰ fot the high N management plots. The $\delta^{15}N$ of the above ground total plant biomass were 2-5 ‰ less enriched than that of the topsoils (Figure 3 and Figure 4). In the high N input four cut treatments the plant $\delta^{15}N$ values increased with increasing N fertilizer application rates except the $\delta^{15}N$ values of *T. repens* L. and *T. pratense* L. with $\delta^{15}N$ signature close to zero, reflecting the $\delta^{15}N$ value of air-derived N as a major N source.

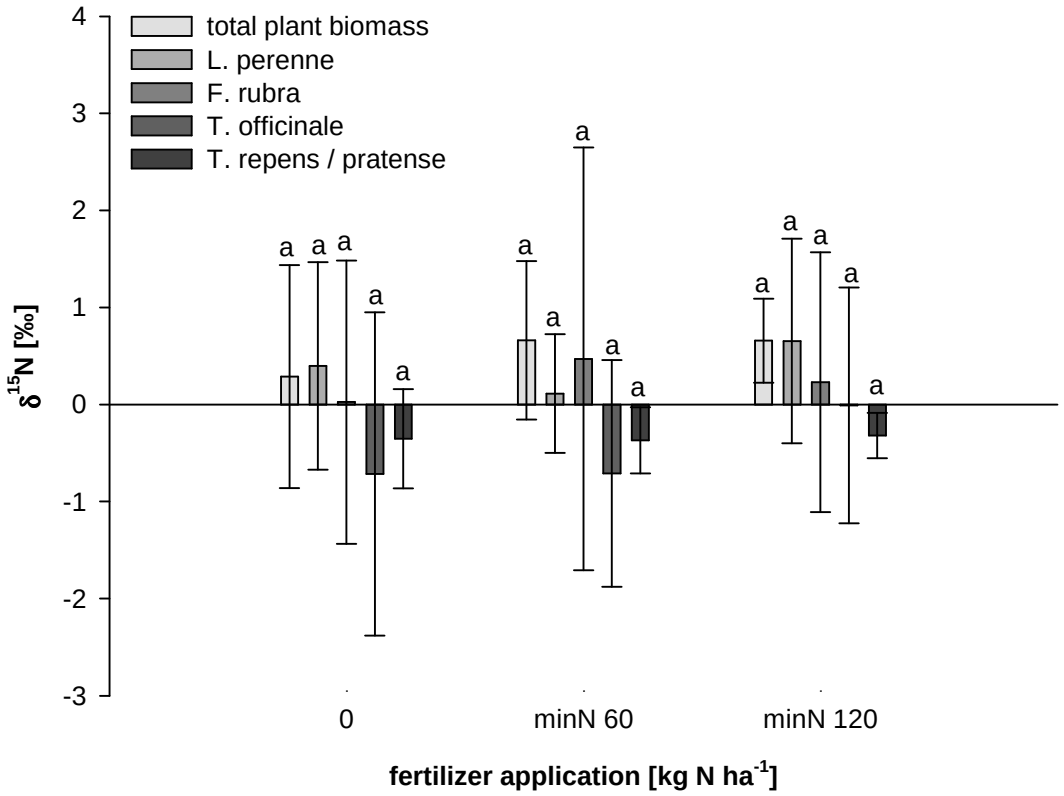

Figure 1: $\delta^{15}N$ abundances of aboveground plant biomass related to (i) the cutting frequency (Low = two cut treatment) and (ii) the amount of fertilizer N applied as mineral N. For abbreviations see Table 4. Five different plant species and total plant biomass were collected in June and September of 2006. Data are exposed as means, each with four replicates per treatment. Values with the same letters refer to no significant differences (one-way ANOVA, $P < 0.05$). Error bars represent the 95 % confidence interval of the means.

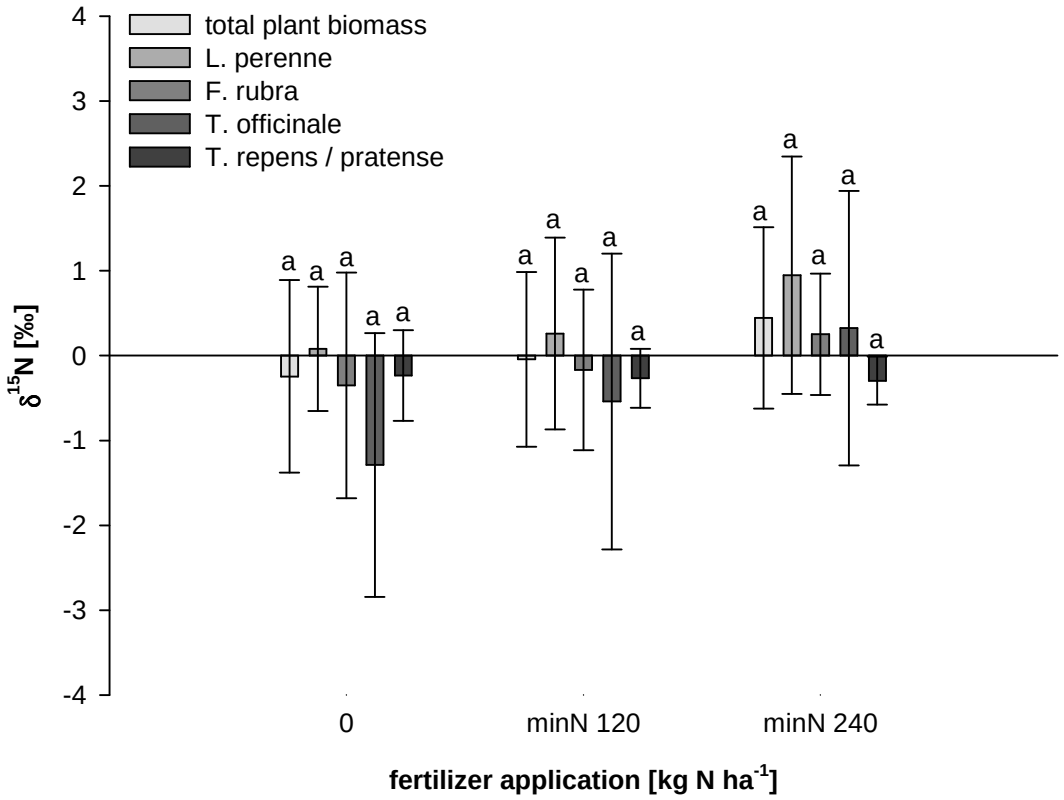

Figure 2: $\delta^{15}N$ abundances of aboveground plant biomass related to (i) the cutting frequency (High= four cut treatment) and (ii) the amount of fertilizer N applied as mineral N. For abbreviations see Table 4. Five different plant species and total plant biomass were collected in June and September of 2006. Data are exposed as means, each with four replicates per treatment. Values with the same letters refer to no significant differences (one-way ANOVA, $P < 0.05$). Error bars represent the 95 % confidence interval of the means.

The $\delta^{15}N$ values of plants in the low N input two cut treatment did not increase constantly with higher fertilizer doses for all plant species and the total above ground biomass. In both treatments the differences of the $\delta^{15}N$ values between the fertilizer applications were negligible and not significant.

2.3.2 $\delta^{15}N$ in soils

Mean values of $\delta^{15}N$ the topsoils (Figure 1 and Figure 2) ranged from 3.2 ± 0.8 ‰ to 3.7 ± 0.2 ‰ for the low N management and 3.1 ± 0.5 ‰ to 3.2 ± 0.6 ‰ for the high N management plots.

The mean $\delta^{15}N$ values of topsoils (0-5 cm) of both varities did not show significant differences between the high and the low N input grassland manangement (low N input: 3.41 ± 0.3 ‰, high N input: 3.14 ± 0.2 ‰). Hence, soils that received mineral N also exhibited elevated $\delta^{15}N$ levels (Figure 3). The results show that the fertilizer isotope signal was not recovered in soil (Watzka et al., 2006). Increasing the amount of chemical-synthetic N fertilization did not resulted in significantly higher $\delta^{15}N$ values of the uppermost soil layer, not in the low nor in the high N input managed grassland. They were not significantly different from that of the control. Hence, natural ^{15}N isotope discrimination likely compensated for the dilution of soil ^{15}N contents by the ^{15}N-depleted mineral N. In most cases, increasing soil depth exhibited higher soil $\delta^{15}N$ contents although this effect was never found to be significant (Figure 3 and Figure 4).

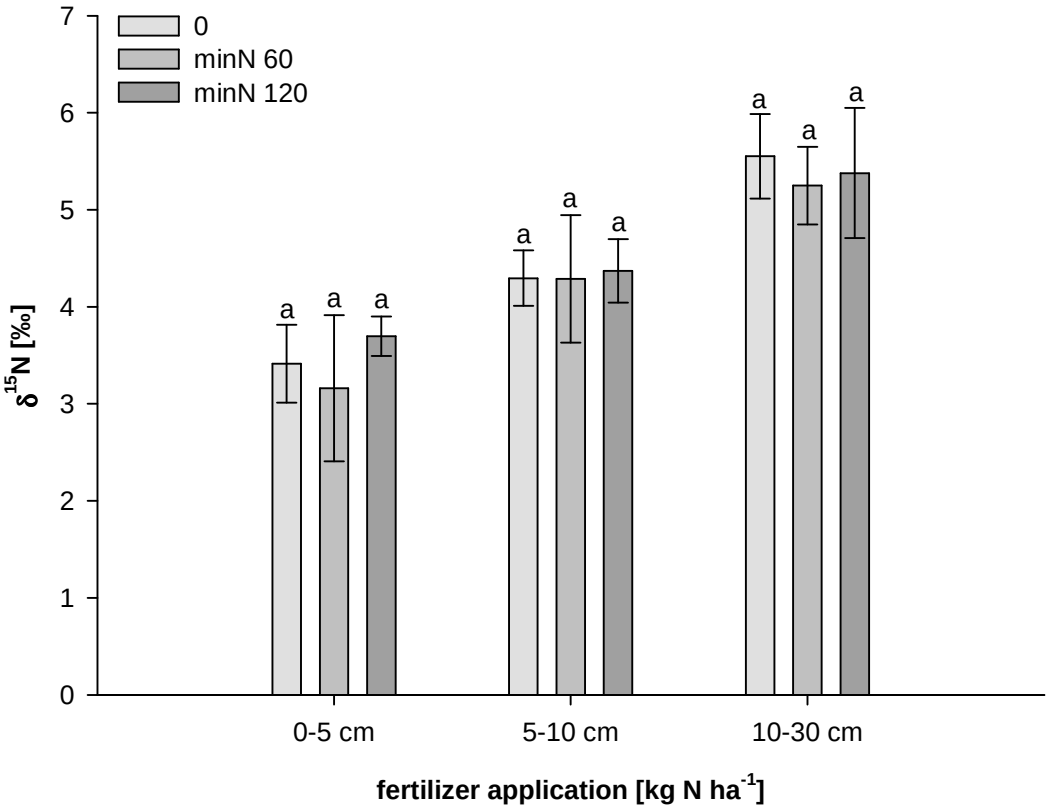

Figure 3: $\delta^{15}N$ abundances of all soil layers (0-5 cm, 5-10 cm and 10-30 cm) as related to (i) the cutting frequency (Low = two cut treatment) and (ii) the amount of fertilizer N applied as mineral N.
For abbreviations see Table 4. The soil samples were collected in June of 2006. Data are exposed as means, each with three replicates per treatment. Values with the same letters refer to no significant differences (one-way ANOVA, $P < 0.05$). Error bars represent the 95 % confidence interval of the means.

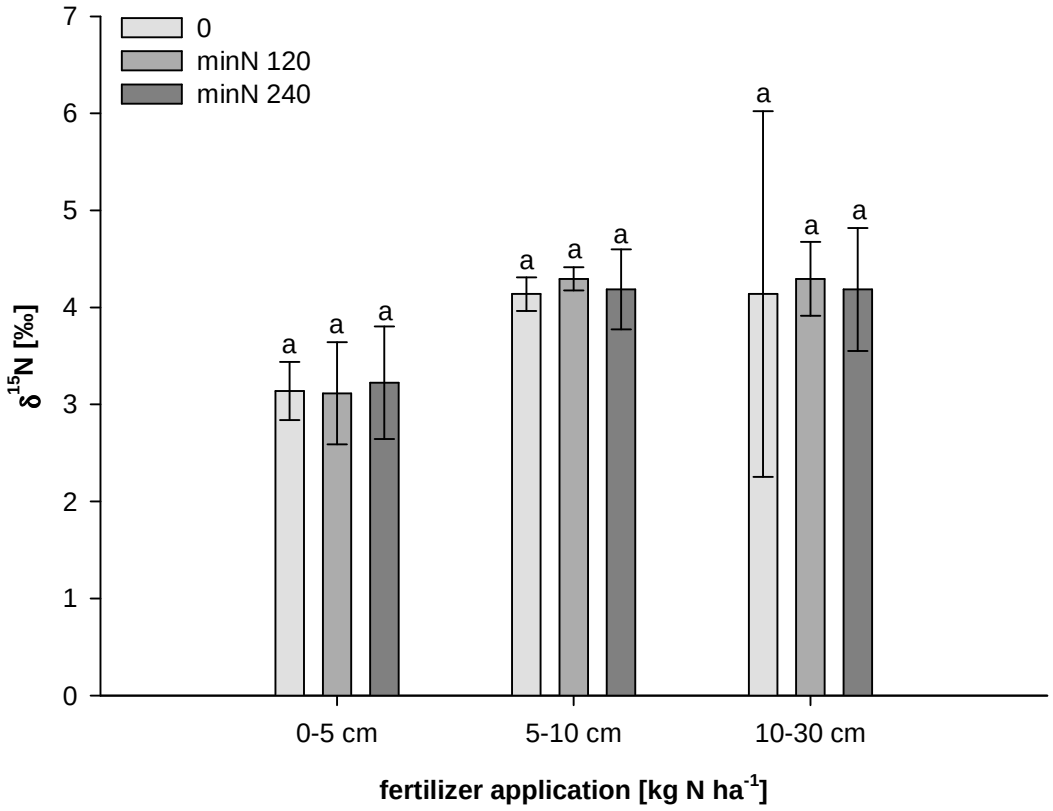

Figure 4: $\delta^{15}N$ abundances of all soil layers (0-5 cm, 5-10 cm and 10-30 cm) as related to (i) the cutting frequency (High = four cut treatment) and (ii) the amount of fertilizer N applied as mineral N.
For abbreviations see Table 4. The soil samples were collected in June of 2006. Data are exposed as means, each with three replicates per treatment. Values with the same letters refer to no significant differences (one-way ANOVA, $P < 0.05$). Error bars represent the 95 % confidence interval of the means.

2.4 Discussion

The N released into soil solution and then absorbed by plants as nitrate-N is usually depleted in ^{15}N. Hence, plants exhibit lower $\delta^{15}N$ values than the underlying soils, as also observed in this study. The $\delta^{15}N$ values of the plants did not show any significant differences between the low and high N input management systems. However, a minor tendency among the plant species could be observed in the high N input management. The variation in $\delta^{15}N$ among different plant species can be attributed to differences in plant growth, resource acquisition strategies or $\delta^{15}N$ values of the soil N pools for which they compete (Nadelhoffer et al., 1996; Dawson et al., 2002).
The $\delta^{15}N$ values ranging within 3.1 ± 0.33 ‰ to 3.7 ± 0.13 ‰ of uppermost soil layer were relatively enriched. Possibly the wet conditions with stagnant water after rainfall

in winter are responsible for the enriched $\delta^{15}N$ values of soil, due to denitrification processes during anaerobic conditions that are characterize with high fractionation factors (see Table 3, Högberg, 1997). The $\delta^{15}N$ values of soils usually increase with soil depth, because of various stable isotope fractionation processes that accompany N uptake and loss (Delwiche et al., 1979; Yoneyama 1996; Gioacchini et al., 2006).
In contrary to the increased $\delta^{15}N$ values of soil samples, the ^{15}N signature of plant was close to zero. It could be assumed that the $\delta^{15}N$ values of plants reflected the isotopic signature of the added mineral fertilizer, since the $\delta^{15}N$ value of chemical-synthetic fertilizer ranged from -1 to +1 ‰ similar to the atmospheric di- nitrogen.
However, there were no signifcant differences of soil and plant $\delta^{15}N$ values between the low N input two cut treatments and the high N input four cut treatments. Furthermore no significant differences of soil and plant $\delta^{15}N$ values between the increasing N fertilizer application rates could be observed.

2.5 Conclusion

If chemical-synthetic N fertilizer is the only applied fertilizer type, it was not possible to use $\delta^{15}N$ values of plant biomass and soil as an indicator of N fertilizer management in grassland systems. The ^{15}N signature of plant biomass and soil did not reflect possible N losses associated with moderate N application rates up to 240 kg N ha^{-1} a^{-1}. It should be tested if $\delta^{15}N$ values are more suitable if additionally organic fertilizer is applied. Furthermore it could be assumed that the amount of N fertilizer input and the amount of accompanied N losses were not sufficient to be reflected by $\delta^{15}N$ values of plant biomass and soil.

3 Long-term changes of the $\delta^{15}N$ natural abundance of plants and soil in a temperate grassland

3.1 Introduction

Testing this hypothesis for a range of different fertilizer trials requires that the $\delta^{15}N$ value of the fertilizer is known. Here, significant differences between fertilizer sources are expected. Urea-derived fertilizer N (Högberg, 1991) and organic farmyard manure is usually enriched in $\delta^{15}N$ (Kreitler, 1979; Wassenaar, 1995; Yoneyama, 1996), mainly because of faster volatilization of the lighter ^{14}N isotopes (e.g. Kerley and Jarvis, 1996). In contrast, chemical-synthetic N fertilizers are generally depleted in ^{15}N, as the N derives from ambient atmospheric di-nitrogen with a $\delta^{15}N$ value close to zero. Hence, analyses of soil and plant $\delta^{15}N$ values may even help to trace back the N fertilizer source (Létolle, 1980; Choi et al., 2003; Bol et al., 2005). Högberg and Johannisson (1993) found elevated $\delta^{15}N$ values after application of high rates of mineral fertilizer N, thereby supporting the hypothesis that the final $\delta^{15}N$ signature has mainly been governed by N losses. Actually, Watzka et al., (2006) confirmed that the $\delta^{15}N$ values of soil and plant materials were closely related to long-term N input-output balances. Yet, there is still a lack of verification of these findings by the direct assessment of the isotopic signature of the N lost within such trials.

Johannisson and Högberg (1994) studied the behaviour of soil and plant $\delta^{15}N$ values along a fertilizer application gradient in order to gain better insight into soil N pool dynamics. They stated that biomass $\delta^{15}N$ analyses are appropriate to indicate short-term changes in the N- balance whereas the $\delta^{15}N$ value of soil rather reflected long-term effects within the large inert (latent) N pool that memorizes pre-treatments. According to Lobe et al. (2005), this latent N pool may be largely associated with mineral soil fractions. The soil $\delta^{15}N$ approached fertilizer ^{15}N values only for the particulate organic matter (POM) of the sand fractions when duration of cultivation increased. To better utilize the $\delta^{15}N$ signature of agroecosystems for the assessment of N balances, it appears necessary to resolve both plant-species effects and ^{15}N fractionation among different plants as well as among different soil organic matter pools and depths.

An elegant way to trace back the N pathways in soil plant- systems relies on the application of ^{15}N labelled fertilizer (Sørensen et al., 1994; Mulholland et al., 2000;

Hatch et al., 2002; Köbl et al., 2006). However, such approaches can hardly be used to analyze the N input-output balance in the long-term because of high costs and limited traceability of labelled ^{15}N after one year of tracer application. The examinations are restricted to small spatial scales (Robinson, 2001). To reconstruct N efficiency in the long-term and at field scales it would thus be helpful to take advantage also of the ^{15}N natural abundance method.

3.2 Materials and Methods

3.2.1 Site description and experimental layout

The experimental site is located at the University Bonn Grassland Research Station in Rengen, in the Eifel Mountains, Germany (50°14'1.23''N, 6° 51'4.20''E) at 475 m.a.s.l. Long-term average annual precipitation was 811mm, and the average annual temperature was 6.9°C (Schellberg et al., 1999).

The soils at the sites were mainly Stagnic Cambisols. However, the lysimeters were located at an 18° slope, where it is to be assumed that the soil was little if at all saturated to the surface throughout the year. Indeed, under the A horizon there is a thin cambic horizon without stagnic colour pattern and stagnic properties did not take place over the sampled 45 cm soil depth. Hence, at least for the topsoil the sites behaved like typical Cambisols only. The soil texture was fine sand to sandy loam with a pH of 5.5.

Data were collected from a field lysimeter experiment that was kept under the same N- treatment for more than 20 years (since 1985). The experiment was established on high N input managed pastures. At the beginning of the experiment floristic composition was dominated by *Lolium perenne* L., *Alopecurus pratense* L, *Dactylis glomerata*, *Taraxacum officinale* Web., *Poa pratensis*, *Festuca rubra* L. The size of individual plots was 2 x 6 m^2. The experiment was cut four times per year at about the same time in May, July, September and October.

The plots were fertilized four times per year at five different N application rates in a randomised complete block design, with three blocks consisting of five permanent grassland plots each. N was either applied as (i) mineral fertilizer (minN) with calcium ammonium nitrate containing 27 % of N, (ii) as organic N with cattle slurry (manN) containing between 2 and 4 kg N m^3 or (iii) as a combination of both. At each application time, the application rates of the organic fertilizers were adjusted to the

exact N content of the treatments. Total annual fertilizer rates ranged from 200 to 480 kg ha^{-1} a^{-1}. In parallel, an unfertilized plot under the same cutting regime served as control. Split doses of the fertilizers were applied at ground level before the growing season and immediately after every cut as described in Table 5.

Table 5: Details of the 22 years old long-term fertilizer experiment setting indicating the amounts (per year and in total), type and $\delta^{15}N$-values [‰] of applied nitrogen (N). All $\delta^{15}N$ values are means ± SE.

Treatment abbreviation	Applied Nitrogen [kg $ha^{-1}a^{-1}$]	Total N applied [kg ha^{-1}]	Type of fertilizer	Total soil N content[1] [t N ha^{-1}]	$\delta^{15}N$ of fertilizer[c] [‰]
0, control	0 (44 fixed N_2)	0 (880)	N_2 fixation (atm.N)	10.38 ± 1.6	0
minN 200	200	4000	Calcium ammonium nitrate[2] (CAN)	10.77 ± 1.4	-1.02 ± 0.2
manN 240	240	4800	Cattle slurry[3]	12.42 ± 3.3	8.88 ± 0.5
minN+manN 360	360 (200+160)	7200	CAN+ Cattle slurry	11.23 ± 1.8	3.38 ± n.d.[4]
manN 480	480	9600	Cattle slurry	12.78 ± 2.9	8.88 ± 0.2

[1] Means± SE (n= 10)
[2] calcium ammonium nitrate (27 % N)
[3] cattle slurry (approx. 2 % N)
[4] n.d = not determined

3.2.2 Sampling and analyses of plant, soil and fertilizer

Bulk soil samples (n= 3) from at least 10 randomized replicates were taken and pooled within each plot at 0-5 cm, 5-10 cm and 10-30 cm soil depth twice each in June and September 2006. Soil samples were sieved to < 2 mm and dried for 48 h at 100°C.

Separate plant samples (n= 3) were taken from 5 species, *Lolium perenne* L., *Festuca rubra* L., *Taraxacum officinale* Web. and a mixed plant sample from *Trifolium repens* L. / *Trifolium pratense* L. Both species of clover (*Trifolium repens* L. / *Trifolium pratense* L.) were analyzed in a preliminary test separately. Plant material of the legume species was pooled in one sample, because their isotopic signature was similar but different from the non-legumes. Additionally, one sample of the total aboveground plant biomass was taken. To avoid edge effects among the plots, all biomass was clipped from the centre of the plots. The pooled samples were cut with a scissor 1 cm above top of mineral soil. All plant material was oven dried for 48 h at

60°C. Samples of both plant and soil material were ground in a ball mill (Retsch, Germany) to achieve homogenous samples prior to ^{15}N analysis.

To approach different soil organic matter pools, two different sand sized SOM fractions were isolated. After mechanical slaking with de-ionised water, the samples were forced through sieves at sizes (Retsch, Germany) of 250 µm and 63 µm. Finally, visibly only POM and sand grains were retained on the sieve so that further ultrasonic dispersion was not necessary. We so received a coarse and a fine sand sized SOM fraction that are known to differ in chemical properties and thus also in turnover rate (Balesdent 1996; Amelung et al., 1999). As sand grains usually contain little if any mineral N, the majority of the N in these sand fractions was likely particulate organic matter in nature (POM; see also e.g Gerzabek et al., 2001; Amelung et al., 1998; Balesdent and Mariotti, 1996). The mineral fractions were not isolated in this study.

After sieving, all samples were oven dried at 100°C to 100 % of dryness and afterwards ground in the ball mill as also done by e.g., Johannisson and Högberg (1993), Kerley and Jarvis (1997) or Watzka et al. (2006). We did not observe a significant N isotope fractionation during this high temperature drying relative to air-dried controls.

Cattle slurry (n= 10) was acidified with H_2SO_4 to pH 5 to avoid N losses through volatilization. Subsequently, all slurry samples were freeze dried and ground in a freezer mill (Freezer Mill 6780; Spex Industries, Edison, NJ, USA). Dry mineral N fertilizer was pulverized without any pre-treatment. All dried samples were weighed into tin cups with a total N content of 15-20 µg N.

3.2.3 Total soil N contents

For calculations of total N content [t ha^{-1}] in soil, bulk density was assessed for the 0-5 cm, 5-10 cm and 10-30 cm depth intervals. It averaged 1.0 g cm^{-3}, 1.1 g cm^{-3} and 1.3 g cm^{-3} for the 0-5 cm, 5-10 cm and 10-30 cm layers, respectively:

$$N\ stock\left[t\,ha^{-1}\right] = bulk\ density \cdot N\left[\%\right] \cdot soil\ layer \cdot 100 \quad . \qquad (6)$$

3.2.4 ^{15}N analysis

All ^{15}N analyses were conducted at the Technical University Munich, Chair of Grassland Science. Isotope ratios of $^{15}N/^{14}N$ and contents of total N in all samples were determined by dry combustion in an elemental analyser (NA 1110; Carlo Erba, Milan, Italy) interfaced with an isotope ratio mass spectrometer (Delta Plus, Finnigan, Bremen, Germany). The isotopic ratios are expressed as $\delta^{15}N$ relative to ^{15}N air standards (Mariotti, 1984):

$$\delta R[‰] = \left[\left(\frac{R_{sample}}{R_{standard}} \right) - 1 \right] \cdot 1000 \quad , \qquad (7)$$

where R is the ratio of the abundance of $^{15}N/^{14}N$ of sample and standard. All samples were measured against laboratory standard gases, which were previously calibrated against the international standard (N_2 in the air) by use of the reference substances N-1 and N-2 for nitrogen isotope ratios provided by the IAEA, Vienna. The analytical precision was 0.2 ‰ for $\delta^{15}N$.

3.2.5 Calculation of Δ15N isotope fractionation and gross N losses

The difference between the theoretical and measured $\delta^{15}N$ values of soils was calculated to demonstrate ^{15}N discriminations ($\Delta^{15}N$) as a result of considerable N losses. The average of three replicates derived from the unfertilized plots served as control.

Assuming that the higher N contents found in the soil after fertilizer application exclusively originated from fertilizer N, thus allowed us to calculate a theoretic $\delta^{15}N$ value ($\delta^{15}N_{calc}$) that consists of both the original soil $\delta^{15}N$ value plus the amount of $\delta^{15}N$ added via the fertilization:

$$\delta^{15}N_{calc}[‰] = \frac{\left(N_{base} \cdot \delta^{15}N_{base}\right) + \left(N_{fert} \cdot \delta^{15}N_{fert}\right)}{N_{measured}} \quad , \qquad (8)$$

and

$$\Delta^{15}N[‰] = \delta^{15}N_{measured} - \delta^{15}N_{calc} \quad , \qquad (9)$$

where N_{base} and $N_{measured}$ are the N contents [t N ha^{-1}] after 20 years of constant management in the control (base) treatment and fertilizer trials. N_{fert} is the cumulative amount of fertilizer added minus the amount withdrawn from the plants (50 % on the

average according to Bristow et al., (1987), Kimura and Kurashima (1991), Fraser et al., (1994), Jenkinson et al., (2004) and yield assessment). The $\delta^{15}N_{base}$ was 1.8 [‰], the $\delta^{15}N_{fert}$ [‰] of the fertilizers are shown in Table 5.

The control plot contained approximately 11 % of N fixing plants of total biomass. We estimated that 4 kg fixed N per percentage of N fixing plants [kg ha^{-1}] have been added to the plots (N_{fixed}; $\delta^{15}N$ =-0 ‰; Table 5) account for this effect. The N losses were thus calculated using the following equation:

$$N_{loss}\left[t\ N\ ha^{-1}\right] = \left(N_{base} + N_{fert} + N_{fixed}\right) - N_{measured} \quad . \qquad (10)$$

With this rough calculation we make the simplified assumption that bulk soil $\delta^{15}N$ fractionations over the years were not significantly influenced by increased abundance of legumes, usually showing little if any $\delta^{15}N$ discrimination during N_2 fixation (Högberg 1997). We thus hypothesize that all marginal fractionation processes that occurred in this plot, take place in plots with N fertilizer application as well, which appeared us to be a justified assumption when considering the large variations in isotope fractionations after N fertilizer application.

3.2.6 Statistics

Statistical analysis was performed separately for each experiment using SPSS 20.0 software (SPSS, Chicago, US). Differences between fertilizer treatments were analyzed by one-way analysis of variance (ANOVA) followed by a multiple range test (Scheffé post hoc test). If homogeneity of variance was missing, Dunnet´s T3 was calculated. For each sample, the 95 % confidence intervals on the mean were computed based on the sample mean and sample standard deviation.

3.3 Results

We only found marginal differences in the analytical results for samples taken at different time intervals (sampling period 1 and 2), so that only the data of the first sample set is shown and discussed below.

3.3.1 δ15N in plants

Mean values of $\delta^{15}N$ of the above ground total plant biomass (Figure 5) were smaller than that of the topsoils (Figure 6 and Figure 7) thus reflecting that the plants discriminated against the heavier ^{15}N isotope at a rate of 1 - 3 ‰. In the control and minN treatments, even negative $\delta^{15}N$ values were recorded, and the plant $\delta^{15}N$ values then significantly increased in the following order: manN 240 ≈ minN + manN 360 < manN 480.

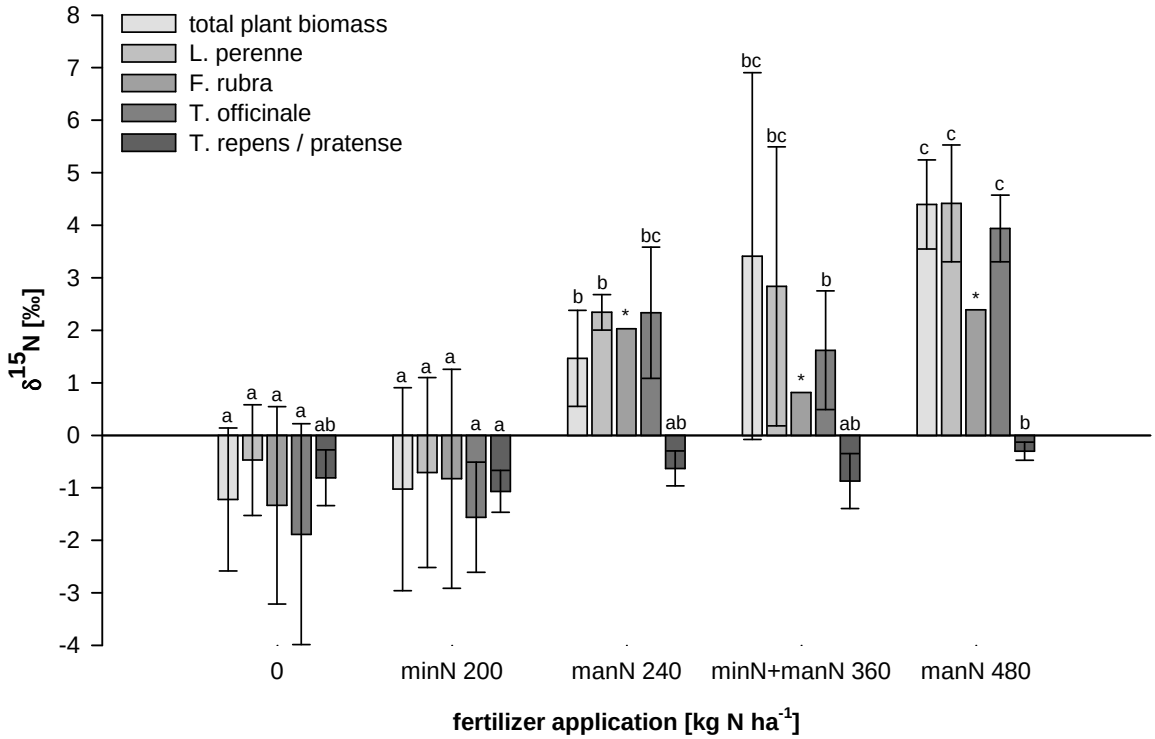

Figure 5: $\delta^{15}N$ abundances of aboveground plant biomass as related to amount of fertilizer N applied as mineral (minN), cattle slurry (manN) and mixed fertilizers (minN + manN). For abbreviations see Table 5. Five different plant species and total plant biomass were collected in June of 2006. Data are exposed as means, each with three replicates per treatment. Values with the same letters refer to no significant differences (one-way ANOVA, $P < 0.05$). Asterisks are used, where less than three replications per treatment were available. Error bars represent the 95 % confidence interval of the means.

The specific plant $\delta^{15}N$ values, however, were correlated with those of the corresponding topsoils only for *Lolium perenne* L., *Festuca rubra* L and *Taraxacum officinale* Web. (Figure 5, Figure 6), but not for *T. repens* L. and *T. pratense* L. The latter kept their $\delta^{15}N$ signature close to zero, reflecting the $\delta^{15}N$ value of air-derived N as a major N source.

3.3.2 Total soil N contents

Among individual plots, total soil N contents varied between 10.4 and 12.8 t N ha^{-1} within the uppermost 30 cm soil layer (Table 5). However, these differences were not statistically significant, i.e., the added fertilizer N did hardly raise soil N contents.

3.3.3 $\delta^{15}N$ in soils

The cattle slurry (manN) was enriched in $\delta^{15}N$ (8.88 ± 0.5 ‰) compared with the bulk soil (1.8 ± 0.2 ‰) and the mineral fertilizer (minN; -1.02 ± 0.2 ‰). Hence, soils that received manN also exhibited elevated $\delta^{15}N$ levels (Figure 6), means that the fertilizer isotope signal was partly recovered in soil (Watzka et al., 2006). Increasing the amount of manN fertilization resulted in significantly higher $\delta^{15}N$ values of the uppermost soil layer, while the effect on the bulk $\delta^{15}N$ signature below 10 cm soil depth was less pronounced (Figure 6).

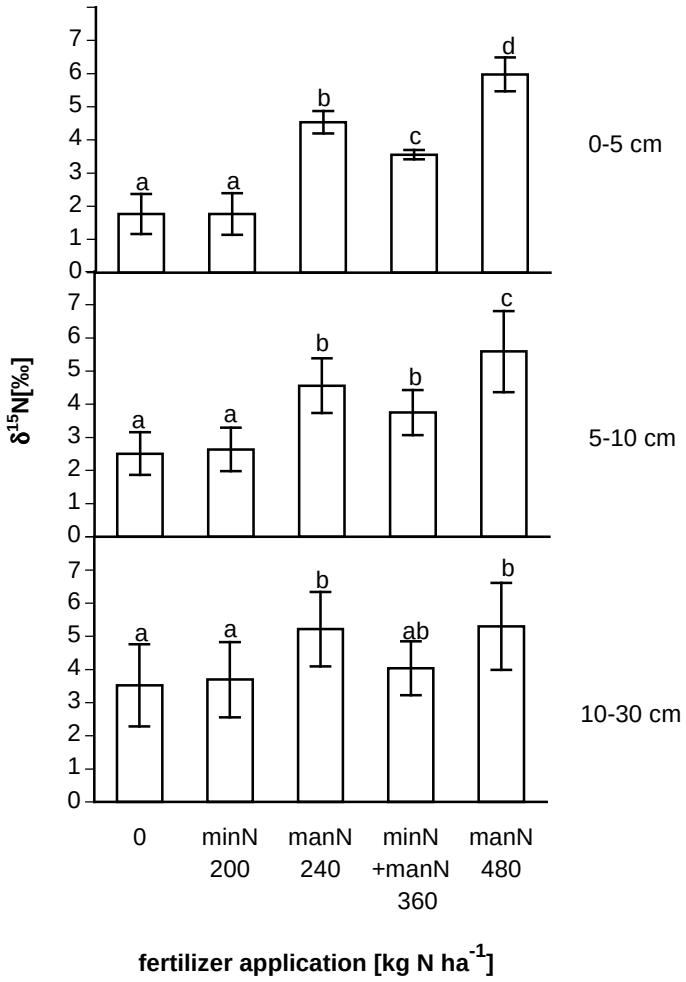

Figure 6: $\delta^{15}N$ abundances of soils related to amount of applied fertilizer N

(for abbreviations see Table 5). Samples were taken out of three soil depths (0-5 cm, 5-10 cm, 10-30 cm) in June of 2006. Data are exposed as means, each with three replicates per treatment. Values with the same letters refer to no significant differences (one-way ANOVA, P < 0.05). Error bars represent the 95 % confidence interval of the means

In contrast to the manN treatments, the soil $\delta^{15}N$ values of the minN treatments were lower. Nevertheless, they were not significantly different from that of the control, despite negative $\delta^{15}N$ values of the minN in the analytical results for samples taken at different depth and sampling periods (June and September 2006). Hence, natural ^{15}N isotope discrimination likely compensated for the dilution of soil ^{15}N contents by the ^{15}N-depleted minN. The mean $\delta^{15}N$ values of soils receiving 360 kg N a^{-1} of both fertilizers (minN + manN 360) were intermediate to both cattle slurry application rates (3.6 ± 0.2 ‰). The manN 480 plots showed the highest $\delta^{15}N$ values and in some layers even at significant levels. In most cases, increasing soil depth exhibited higher soil $\delta^{15}N$ contents although this effect was never found significant (Figure 6).

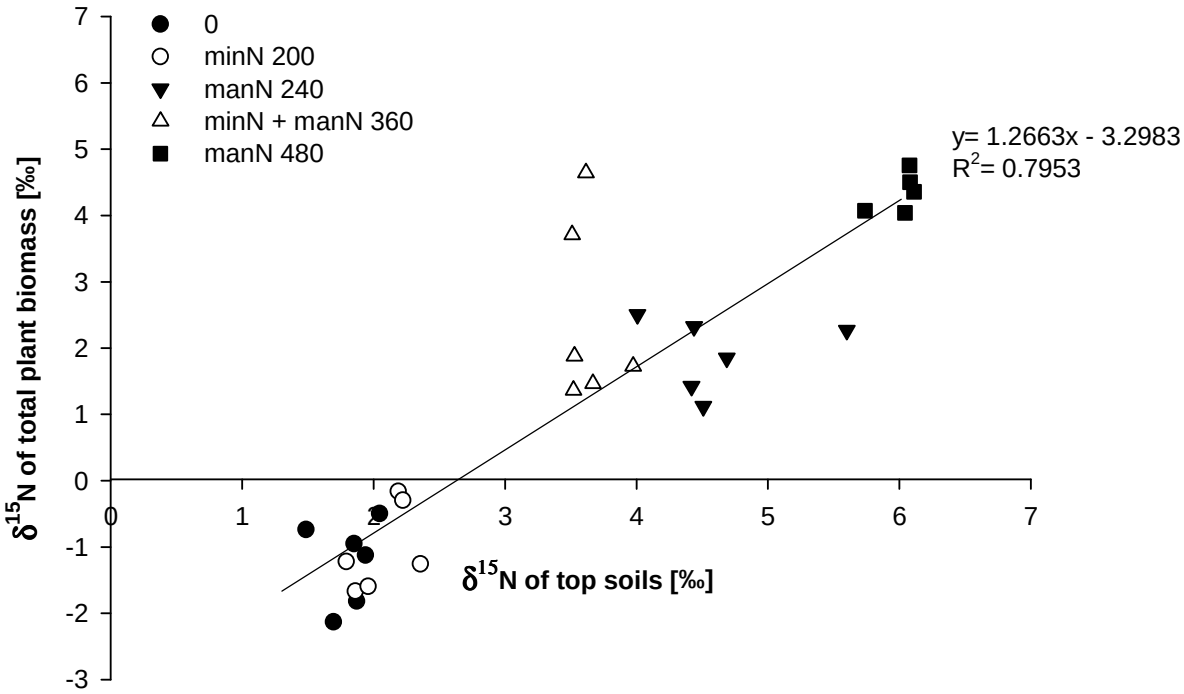

Figure 7: Relation between $\delta^{15}N$ of topsoils and the $\delta^{15}N$ of removed aboveground total plant biomass in 2006.
Data are presented as single values for each pooled sample showing a significantly high coefficient of determination (R^2: 0.795; P< 0.05). For abbreviations see Table 5.

3.3.4 $\delta^{15}N$ of the sand sized SOM fractions

Both sieved sand sized SOM pools showed positive $\delta^{15}N$ values in each treatment (Figure 8). Again, fertilizer inputs were indicated by increasing $\delta^{15}N$ values, especially in the manN treatments. In contrast to bulk soil (Figure 6) these effects are now clearly seen also in the subsoil (Figure 8). Relative to the $\delta^{15}N$ values of the bulk soils and above ground total plant biomass, the $\delta^{15}N$ of both sand sized SOM fractions were thus intermediate, indicating that this fraction is an intermediate pool from plant debris to SOM (soil organic matter) formation. And indeed, the $\delta^{15}N$ coarse sand size SOM fraction was closer to the total plant biomass, whereas that of fine sand sized SOM was closer to that of soil. As soil depth increased, also the $\delta^{15}N$ values of sand sized SOM fractions increased.

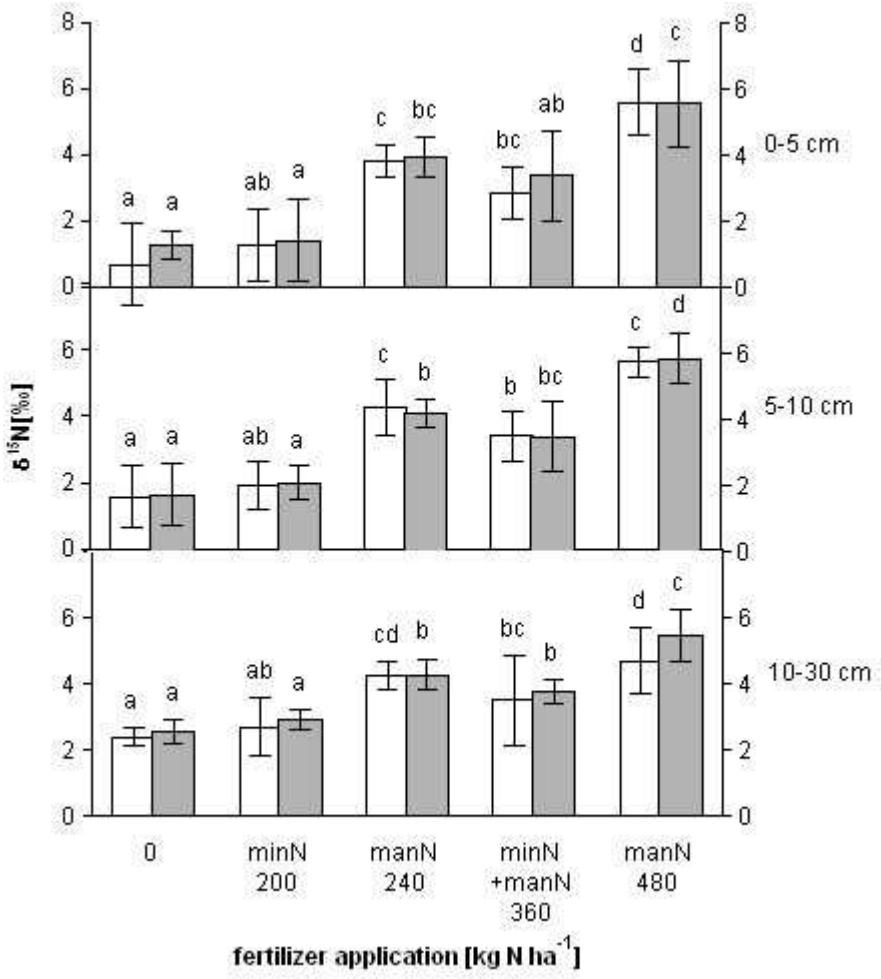

Figure 8: $\delta^{15}N$ abundances of coarse (≥ 0.25 mm; open bars) and fine sand sized soil organic matter (≥ 0.063 mm; closed bars) related to amount of applied fertilizer N (for abbreviations see Table 5). Samples (n= 3) were taken out of three soil depths in June of 2006.

3.3.5 Estimation of $\Delta^{15}N$ and N losses

Calculated N losses of the uppermost soil layer (0-5 cm) increased with increasing amounts of N fertilizer application (Figure 9). The N losses ranged from 0.4 to 4.5 [t N ha^{-1}] in the order 0 < minN 200 < manN 240 < minN+manN 360 < manN 480. The calculated $\Delta^{15}N$ values correlated with the $\delta^{15}N$ of soils and plants. Differences between the calculated and measured N increased from 0.01 to 1.48 [‰] with an intermediate $\Delta^{15}N$ value for the minN + manN 360. Consequently, the measured $\delta^{15}N$ values were throughout higher than the calculated soil $\delta^{15}N$ values. This calculated discrimination effect can also be found in deeper soil layers (0 - 30 cm, data not shown) but with lower intensity, due to thinning effects.

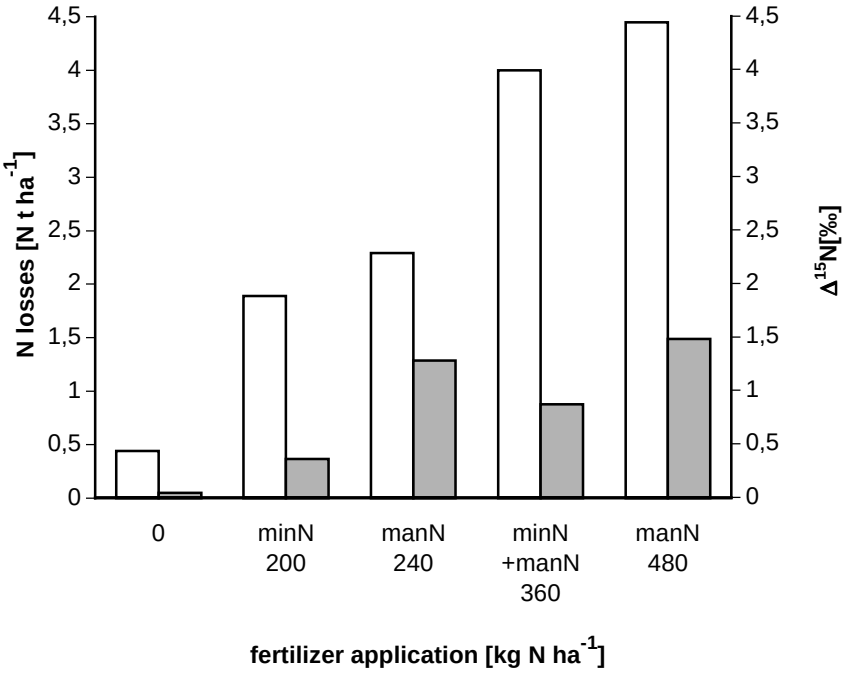

Figure 9: Calculated N losses (open bars) and calculated $\Delta^{15}N$ fractionation (closed bars) of topsoils (for abbreviations see Table 5) since 1985.

3.4 Discussion

Within the 22 years of the experiment, up to 10 t N ha^{-1} have been added to the soils (manN 480). Edmeades (2003) reported that fertilization with cattle slurry rather than with mineral N may increase soil N contents, because even at similar nutrient level a larger portion of N is preserved in the additionally accumulating source of organic matter. In our study, a trend to higher N contents in cattle slurry amended soils was

found as well, but they did not differ significantly within all treatments (Figure 6 and Figure 7). This result agrees with observations from the Askov long-term experiments on arable land (Bol et al., 2005). We conclude, therefore, that the added N was thus either taken up by plants and removed by continuous cutting or has been lost to atmosphere and soil (Watzka et al., 2006). So, all processes involved have led to ^{15}N isotope discrimination. With annual herbage yield harvested of up to 9 t ha^{-1} and an N uptake rate of about 50 % (Bristow et al., 1987; Kimura and Kurashima, 1991; Fraser et al., 1994, Jenkinson et al., 2004), up to 50 % of the added fertilizer N was not quantitatively retrieved in the surface soil. Hence, natural $\delta^{15}N$ isotope abundance measurements are needed to trace back the fertilizer history, allowing also to test our central hypothesis that increased N losses are reflected in increased $\delta^{15}N$ fractionation.

3.4.1 Plant-specific effects of $\delta^{15}N$ fractionation

In the lysimeter experiment the $\delta^{15}N$ values of the plants were strongly influenced by the underlying management. In contrast to the preceding trial (Chapter 1) the $\delta^{15}N$ values of the plants depended on both, the N fertilization treatment and plant species. The effect of the former was reflected by the close correlation between plant and soil $\delta^{15}N$ (Figure 7). As already assumed differences in plant growth, resource acquisition strategies or $\delta^{15}N$ values of the soil N pools for which they compete (Nadelhoffer et al., 1996; Dawson et al., 2002) may result in different ^{15}N signatures of plants. In this experiment *Lolium perenne* L. was always characterized by higher $\delta^{15}N$ values than *Festuca rubra* L., for instance, likely reflecting a different functional N uptake efficiency. *Lolium perenne* L. tends to take up N at higher rates compared with *Festuca rubra* (Schulte auf´m Erley, 2001). *Taraxacum officinale* Web. on the other hand had the highest $\delta^{15}N$ values of all sampled species and was the most deeply rooted species. We suggest that this species competes most effectively for N in deeper soil profiles among the species observed here, as e.g., Johannisson (1996) also reported for *Eriophorum* and *Carex* species. The $\delta^{15}N$ values of *Trifolium repens* L and *Trifolium pratense,* two N_2-fixing species, were close to 0 ‰, suggesting that much of their N requirement was accomplished by fixation of atmospheric nitrogen. At high fertilizer loads, however, also the $\delta^{15}N$ values of these plant species increased, reflecting that N fixation is reduced at N surplus from fertilization (Paynel et al., 2008), and that most of the utilized N derived from the soil N pools. Hence, these plant species may be sensitive indicators of N surplus in these pasture ecosystems.

Looking at the magnitude of plant $\delta^{15}N$ values it becomes obvious that the negative values were detected in the control and minN 200 fertilization trials. Thus, the plant $\delta^{15}N$ mainly reflected the ^{15}N signal of mineral N that they absorbed. Choi et al., (2003) suggested that the $\delta^{15}N$ of plants was largely affected by N derived from compost or mineral fertilizer, rather from soil N. In our study the $\delta^{15}N$ of plants sampled from the manN 240, minN+manN 360 and manN 480 plots, however, were too low to be influenced solely by the high enriched cattle slurry, especially when taking into account that volatilization of NH_3 additionally increased the $\delta^{15}N$ value of the slurry in these trials (see above and Table 5). Hence, we conclude that in this grassland a major part of plant-N also originated from the soil rather than from the fertilizer N. Possibly there was a priming effect of soil N released by high organic N amendments, as earlier also reported for soil C (Bol et al., 2000; Bol et al., 2003; Kuzyakov, 2005).

3.4.2 $\delta^{15}N$ isotope discrimination in soil

The $\delta^{15}N$ values of soils usually increase with soil depth, because of various stable isotope fractionation processes that accompany N uptake and loss (Delwiche et al., 1979; Yoneyama 1996; Gioacchini et al., 2006). Nevertheless, within the top 30 cm of soil, the fractionation processes were negligible pronounced (Figure 6), thus interfering little with the detection of fertilizer $\delta^{15}N$ values within these samples. The results of this work confirme those of Watzka et al. (2006) indicating that long-term fertilizer application had a significant effect on the $\delta^{15}N$ values of both the soil and standing plant biomass. In particular, two factors influenced this $\delta^{15}N$ signature, namely the type and the amount of applied fertilizer.

The applied calcium ammonium nitrate is commonly produced from atmospheric di-nitrogen ($\delta^{15}N$: ~0 ‰) by the Haber-Bosch technique, and hence exhibits a $\delta^{15}N$ value of approximately -1 ‰ (Shearer et al., 1974; Wassenaar, 1995). In contrast, cattle slurry is enriched in ^{15}N, due to fractionation processes during digestion and storage (Jarvis and Kerley, 1996; Choi, 2002). Our $\delta^{15}N$ values for manN plots were thus within the range of $\delta^{15}N$ values earlier reported for animal manure (6 - 13 ‰, Wassenaar, 1995; Glaser et al., 2001; Choi, 2002; Bol et al., 2005; Watzka et al., 2006). Hence, with increasing portions of organic N applied, $\delta^{15}N$ values increased for both plants and soil, thus corroborating with earlier findings of long-term studies, with an experimental run-time up to 50 years e.g. Yoneyama (1996), Choi et al. (2003) and Watzka et al. (2006). Further, higher fertilization rates resulted in elevated

$\delta^{15}N$ values for soils. Noteworthy, also the mineral fertilizer treatments did not significantly reduce the soil $\delta^{15}N$ value (Figure 6), despite the negative $\delta^{15}N$ signal of the N input (Table 5). Since there will always remain some residual fertilized N in the soil (Jenkinson et al., 2004), I conclude that N discrimination processes occurred with fertilizer N and so the heavier ^{15}N isotopes remained. To highlight the underlying processes, I compared the theoretical $\delta^{15}N$ signatures from the fertilizer additions recovered in SOM with the measured ones according to Equation 8.

Actually the difference between measured $\delta^{15}N$ and calculated $\delta^{15}N$ were positive in each treatment (Figure 9) which indicates that ^{15}N fractionation occurred during the incorporation of the fertilizer N into the soil. This fractionation was highest for the highest N input treatments, in the manN 480 plots. Different processes may account for this. Particularly high $\delta^{15}N$ fractionation rates are reported for ammonia volatilization (0 - 29 ‰), nitrification (0 - 35 ‰) and denitrification (0 - 33 ‰) processes (Shearer and Kohl 1986; Handley and Raven, 1992; Högberg, 1997). The mean N losses through NO_3 leaching, measured in the years 2003 to 2005 were only marginal (< 5 kg a^{-1}). In previous studies by Stratmann (1989), Paaß (1993) and Anger (2001, 2005) values in the range of 3, 6 -11 and 5 - 15 kg ha^{-1} a^{-1} for N losses through NO_3 leaching were reported. Stagnant water regime might have favoured N losses through denitrification. However, total N_2O losses in this investigation had almost no effect on total N balances. Total isotope fractionation in the minN treatments was low (Figure 6), indicating that also denitrification did not play a major role in total ^{15}N discrimination. I therefore suggested that the N losses through NH_3 volatilization were the most important parameter influencing $\delta^{15}N$.

Evans (2001) and Robinson (2001) assumed that the ^{15}N isotope technique might not be applicable to quantify surplus situations on grassland fields or farms. While this may remains true for short-term fertilization trials, however, this data clearly show that application of excess N over a long period is at least detectable using natural $\delta^{15}N$ abundance measurements. Yet, most of the added fertilizer ended in the surface near soil horizons, with little additional increase in $\delta^{15}N$ from the top 10 cm to lower soil depths, except in the manN 480 treatments. Apparently, the cattle slurry signature was not detected at lower soil depths, presumably reflecting the much higher stocks of soil-inherent N.

A large part of the soil N is bound in stable forms of the organic N, thus hardly contributing to the short-term cycling of N during the season (Jansson,1985). There may be an exchange between active and passive soil N pools though (Johannisson

and Högberg 1994), which may show elevated $\delta^{15}N$ values as a result of losses of ^{15}N depleted mineral N, produced for instance during nitrification, denitrification, ammonia volatilization and leaching (Shearer and Kohl 1986; Handley and Raven 1992). In this study I did not investigate the active soil N pool such as living soil microbial biomass, which only exhibits approximately 2 % of total N (Perakis and Hedin, 2001). If all fertilizer N applied throughout 22 years would have ended in the active pool, it would have hardly been able to detect changes in the total soil $\delta^{15}N$ value. Recovering the fertilizer signal in the total N pool, therefore, gives support to the hypothesis that a significant fraction of the applied fertilizer N has already entered into the more stable N forms. On the other hand I must not assume that a complete replacement of the soil organic N pool by fertilizer N takes place even within this long experimental period. Instead, the turnover rates of individual N-containing SOM fractions frequently reach decades to several hundred years or even longer (Trumbore et al., 1989; Balabane and Balesdent, 1995; Amelung et al., 2006).

To trace back the pathway of fertilizer ^{15}N into stable SOM pools, I isolated two different SOM fractions. In general, the turnover of SOM increases with increasing particle-size in the order coarse sand sized SOM > fine sand sized SOM > mineral associated SOM. Partly, because the finer-sized SOM contains debris from the degraded coarser fractions (e.g. Balesdent, 1996; Amelung et al., 1998). The increase in $\delta^{15}N$ signals in the order plant biomass < coarse sand sized SOM < fine sand sized SOM < bulk soil thus reflected the increased $\delta^{15}N$ isotope fractionation during soil organic N genesis. Fine SOM fractions containing more products from plant debris and are therefore correlated with the age of the nitrogen. Hence the fine sand fraction is much older than coarse sand fraction.

With increased amounts of fertilizer N, also the $\delta^{15}N$ values increased in both SOM fractions, suggesting that a significant part of the added N already affected also SOM pools such as fine SOM with an estimated turnover time of < 15 years (arable soil; Balesdent and Mariotti, 1996) or even longer (due to the lack of ploughing in the grassland). Hence, it has not been the short but the long-term cycling of fertilizer N that affected soil $\delta^{15}N$ values.

3.5 Conclusions

The $\delta^{15}N$ values of soils and above ground biomass allowed us to trace back the source of the fertilizer and also the efficiency of the fertilizer management. Higher N surplus resulted in higher $\delta^{15}N$ values of soils and plants, because mainly the lighter

^{14}N was lost from the grassland ecosystem. While the history of the amount and kind of the N fertilization treatment left kind of a footprint in the SOM the differences in the N- uptake by the vegetation cover was reflected in plant specific $\delta^{15}N$ variations only for the short-term.

4 Revealing N management intensity on grassland farms based on natural $\delta^{15}N$ abundance

4.1 Introduction

Stable isotopic approaches are increasingly used to study the impact of environmental change and agricultural practices on ecosystems (Kerley and Jarvis, 1996; Dittert et al., 1998). The natural abundance of the stable isotope of ^{15}N ($\delta^{15}N$) provides insight into the main N transformation processes in the N cycle (Högberg, 1997; Robinson, 2001). This is attributed to the isotopic discrimination of heavy against light isotopes in biochemical processes due to their mass differences. Every process resulting in N losses thus discriminates against the heavier isotope ^{15}N. This causes a depletion of ^{15}N in products that are lost to the environment (NH_3, N_2, NO, NO_3^-) and an enrichment of ^{15}N in the residual substrate (NH_4^+, organic N).

Long-term forest fertilization trials showed elavated $\delta^{15}N$ isotope ratios in needles, grass and soil samples (Högberg, 1990; Högberg and Johannisson, 1993; Johannisson and Högberg, 1994; Templer et al., 2007), mainly due to the greater losses of the lighter ^{14}N isotope through volatilization of ammonia (NH_3), leakage of NO_3^- and denitrification (Högberg and Johannisson, 1993; Johannisson and Högberg, 1994; Templer et al., 2007). These observations have been increasingly confirmed by investigations on cropland (Meints et al., 1975) and grassland (Frank et al., 2004; Watzka et al., 2006; Kriszan et al., 2009; Wrage et al., 2011). The degree of ^{15}N enrichment depends on type and amount of fertilizer used (Kriszan et al., 2009). Among them, organic amendments, such as animal manure, slurry or compost are enriched in ^{15}N (Kreitler, 1979; Wassenaar, 1995; Yoneyama, 1996), and so are the soil and plants which received this fertilizer (Watzka et al., 2006; Yuan et al., 2012). In contrast, N in chemical-synthetic N fertilizers originates from atmospheric N synthesized in Haber-Bosch procedure. This is why $\delta^{15}N$ signature of soil and plant material typically remains depleted or unaffected when these fertilizers are applied and why their $\delta^{15}N$ values consistently range between -2 and +2 ‰. From these findings one can conclude that stable isotope analyses of ^{15}N also can serve as a suitable indicator that allows distinguishing between conventionally and organically agricultural management, due to the exclusive use of farm manure in organic farming

systems (Schmidt et al., 2005; Bateman et al., 2007; Flores et al., 2007; Bahar et al., 2008; Yuan et al., 2012). Thus, $\delta^{15}N$ should also be a suitable indicator for reconstructing the intensity of N management on practice farms.

It has been shown, that the N signature of animal products such as milk is also influenced by the $\delta^{15}N$ values of the feed with different degrees of fractionation during digestion (DeNiro and Epstein, 1981; Kornexl et al., 1997; Knobbe et al., 2006). Samples like blood, fat and liver memorize the diet at a range of weeks to month, whereas animal tissues like hair or bones are suitable to integrate the dietary information across longer time scales due to low metabolic activity. Urine and faeces are less studied than hair, although urine also allows conclusion on diets (Sponheimer et al., 2003; Knobbe et al., 2006). Sampling of milk, urine faeces and slurry and the preparation for stable isotopic analysis is simple, i.e., these materials could therefore additionally help to trace back the history of the underlying N management.

The objective of this study was to relate $\delta^{15}N$ isotope ratios of several N pools of grassland farms to the underlying N management of the latter. I hypothesized that the isotopic signature of the N pools reflects long-term N losses, which in turn are influenced by the N application technique and the distance between farmyard and pastures or meadows.

4.2 Materials and Methods

4.2.1 Study area

The study area was located between 50°13’ to 51°27’ N and 6°12’ to 8°51’ E in North Rhine Westphalia and Rhineland- Palatinate, Germany, and included sites between 260 m and 520 m above sea level. Additionally, one site in Styria, Austria was chosen (47°29’ N and 14°06’ E, 900-1100 m above sea level). Long-term mean annual air temperatures ranged between 7° and 10° C. Long-term mean annual precipitation varied between 830 and 1200 mm. Soils were predominantly of sandy loamy texture and comprised mainly Cambisols and Stagnosols (WRB, 2006) with pH values ranging from 4.6 to 7.0 (data provided by farmers).

4.2.2 Farms selection

With the support of local agricultural services, nine typical livestock farms were selected. Farms denoted as A, B, C, E, G, H and I were typical grassland farms with a proportion of grassland of more than 90 %, whereas farm D cultivated arable crops on approximately 16 % of its total farm land. Merely, the bull fattening farm (F) had a higher proportion of arable land (71 %). Based on the production system we separated the farms into five conventional (A, B, C, E, F) and four organic (D, G, H, I). On four farms (F, G, H, I), some fields were managed following the nature conservation program (NPA= nature protection by agreement) funded by the government. Fertilizer application is not permitted on these NPA fields. Farm (I) was characterized by a very low N input, a low milk production rate and a more integrated and self contained system due to its exposed location in the Austrian alpine region.
Farm data and samples were collected twice in 2007, before (April/May) and after the grazing season (October/November). On farm H sampling of biomass and soil on three out of eight specific fields were carried out only once in April/May. On farm I all samples were only taken once (July). In addition, we recorded data on farm management characteristics and nutrient bookkeeping from interviews and stated questionnaires (see Table 6).

Table 6: Production characteristics of the studied grassland farms (A-I) in 2007.

	Individual Farm								
	High N input (HNI)			**Low N input (LNI)**					
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**
Production type	Dairy	Dairy	Suckler cow	Dairy	Dairy	Bull fattening	Dairy	Suckler cow	Dairy
Production systems characteristics	Conv.[1]	Conv.	Conv.	Organ.[2]	Conv.	Conv.	Organ	Organ	Organ
NPA[3] fields (No. of plots)	No	No	No	No	No	Yes (1)	Yes (1)	Yes (2)	Yes (1)
Cattle keeping	Confine-ment[4]	Confine-ment	Confine-ment	Summer grazing	Summer grazing	Summer grazing	Half-day grazing	Summer grazing	Summer grazing
Slurry application technique [LE/HE][5,6]	LE	HE	HE	HE	LE	HE	LE	LE	HE
Total milk production [t a^{-1}]	2,600	1,450	n.a.[7]	0,350	0,190	n.a.	0,905	n.a.	0,051
Milk yield [l cow^{-1} a^{-1}]	8700	9800	n.a.	5800	5100	n.a.	7500	n.a.	4600
Stocking rate [LU ha^{-1}][8]	3.7	2.1	4	0.9	1.2	0.5	1.4	1	1
N input/output balance[9] [kg N ha^{-1} a^{-1}]	196	24	102	-111	-51	-130	-110	-102	-99
Precipitation [mm]	850	1150	1150	900	1200	800-1000	730	800	1100
Average Temperature [°C]	9.7	7.1	7.8	8.5	8	7.8	7.5	6.9	6.8
Location [m a.s.l.][10]	250	450	350	250-350	420	300-400	540	475	1000

[1] = Conventional farming
[2] = Organic farming
[3] = nature protection agreement (no additional use of fertilizer N)
[4] = Year-round indoor housing of animals
[5] = LE= low emission
[6] = HE= high emission
[7] = not available
[8] = Livestock unit per agricultural area (= 500 kg live weight)
[9] = N-balance was calculated using detailed information provided by the chamber of agriculture and on additional information given by the farmers
[10] = m.a.s.l.= meters above sea level

4.2.3 Nitrogen balances

N balances were calculated based on the data provided by the North Rhine-Westphalia (NRW) Chamber of Agriculture and on additional information provided by the farmers. The N input comprised applied mineral and organic N fertilizer, atmospheric N_2 fixation by legumes and NH_3 deposition. Total N fertilizer imports to the grassland reached 450 kg N ha^{-1} a^{-1} on the HNI farms, 12 to 48 % of which were applied as calcium ammonium sulphate. On LNI farms, 100 % of fertilizer N was derived from animal excreta. Across all farms, the swards consisted of 0 to 40 % legumes, in most cases approximately 10 - 15 %. The N input via N_2 fixation was determined for each field by multiplying percentage dry matter contribution (DM %) of legumes in the sward by annual N_2 fixation capacity (3 kg N ha^{-1} a^{-1}) (Pötsch 1998). In addition to N fertilizer application and symbiotic fixation of atmospheric N, we took into account an estimated 20 kg N ha^{-1} a^{-1} of deposited NH_3 (Schwertl et al., 2005; Watzka et al., 2006). The N-exports were estimated from the official field-scale nutrient balance, mainly in the form of biomass production, selling of organic N fertilizer and N losses during storage and application. Further, individual and more detailed information about the N management of each field plot provided by the farmers were taken into account to specify more precisely the field-scale nutrient balance.

4.2.4 Preparation of samples

On each farm, plant samples were taken from five subsites on five or seven (farm H) selected meadows or pastures providing the bulk forage production of the farm, leading to a sample size of n = 25 per farm. The fields differed considerably in the distance to the farmyard ranging from 50 to 5500 m. We created three classes close (average distance = 100 m), intermediate (average distance = 900 m) and far (average distance = 2200 m).

One pooled bulk soil sample from at least 10 randomized replicates was taken at 0-5 cm soil depth from each of the five fields in question (i.e. n = 5 per farm). Soil samples were sieved to < 2 mm and dried for 48 h at 100°C. At each subsites, five samples of plant biomass were cut with scissors 1 cm above ground. Pooled samples of animal feed, such as grass silage and hay (not for farm B and H) were taken from each farm. All plant biomass and feed samples were oven dried for 48 h at 60 °C.

Thereafter, feed samples and soil material was ground in a ball mill (Retsch, Germany) to obtain homogenous samples prior to ^{15}N analysis. Hair samples were collected from three cows (Deutsche Schwarzbunte, Deutsches Fleckvieh, Limousin) per farm except for farm F and H. A tuft of tail hair (5-10 cm) was cut with scissors as close as possible to the skin (root of hair). These samples were prepared according to Schwertl et al. (2003). Contaminations of hair with faeces were removed by ultra-sonication with de-ionised water. After drying for 48 h at 40°C, they were soaked in a 2:1 (v/v) mixture of methanol/chloroform solution to remove surplus fat and then dried again under the same conditions. Thereafter, an aliquot was ground in the freezer mill (Freezer Mill 6780; Spex Industries, Edison, NJ, USA) prior to $\delta^{15}N$ abundance measurements.

Samples of urine (not for farm C and F) and faeces were collected from at least three cows per farm. One pooled sample of cattle slurry was taken and, for farm D, E, H and I, a bulk sample of farmyard manure was collected at each sampling period. To avoid N losses through volatilization, samples were immediately acidified with H_2SO_4 to pH 4 - 5. Additionally, on the dairy farms (A, B, D, E, G, I) one pooled sample of milk was taken. Subsequently, all samples were freeze dried and ground in a freezer mill (Freezer Mill 6780; Spex Industries, Edison, NJ, USA).

4.2.5 Isotope ratio measurements

The dried samples were weighed into tin capsules and combusted in an elemental analyser (NA 1110; Carlo Erba, Milan, Italy) interfaced (Con FLo III, Finnigan MAT, Bremen, Germany) to an isotope ratio mass spectrometer (Delta Plus, Finnigan MAT). Nitrogen isotopic data was represented as $\delta^{15}N$ (‰) relative to the air nitrogen standard:

$$\delta R[‰] = \left[\left(\frac{R_{sample}}{R_{standard}} \right) - 1 \right] \cdot 1000 \quad , \qquad (11)$$

where R is the ratio of $^{14}N/^{15}N$. All samples where measured against laboratory working standard gases, which were previously calibrated against IAEA secondary standard (IAEA No. 3).

4.2.6 Statistical analysis

All results were evaluated using analysis of variance (ANOVA) with the software package SPSS v. 20.0 for windows software (SPSS, Chicago, US), except for comparisons of $\delta^{15}N$ values of various N pools (Figure 1) and for $\delta^{15}N$ values in top soils and plant biomass in relation to the N fertilizer application and application technique (without NPA). ANOVA was followed by a multiple range test (Scheffé *post hoc* test). If homogeneity of variance was missing, Dunnet´s T3 was calculated. For each sample, the 95 % confidence intervals on the mean were computed based on the sample mean and sample standard deviation. Hierarchical clustering of $\delta^{15}N$ values of soil and biomass of all farms was done with SPSS software 20.0 (IBM® SPSS® Statistics). Between groups linkage method was applied, and squared Euclidean distance was selected as measurement.

4.3 Results

4.3.1 Isotope signatures of various N pools and the impact of N balances on $\delta^{15}N$ values

Pooled $\delta^{15}N$ values of samples taken in late spring did not differ significantly from those sampled in autumn 2007 ($p \geq 0.05$). Hence, the values of both periods were merged and were further considered as mean values in the following (see Table 7). Large variations occurred among the sampled N pools. This is most evident from the $\delta^{15}N$ values observed for urine and slurry, which differ by almost 17 ‰. Urine was constantly the pool that was most depleted in ^{15}N (-2.94 ‰). Its $\delta^{15}N$ values remained negative even on farms which applied highest amounts of total N fertilizer. As expected, the highest $\delta^{15}N$ values were detected in the slurry (+13.78 ‰).

Based on their $\delta^{15}N$ values, the N pools could be divided into four groups: I. soil II. feed, III. animal products, and IV. animal excreta (Figure 10 and Table 7). Except for the latter, isotopic signatures did not differ significantly within the groups (Table 7). Moreover, there was a significant difference between farms managd at low and high N fertilizer regime. On HNI farms, all N pools were enriched in ^{15}N and the respective $\delta^{15}N$ values were significantly elevated by 1.46 (urine) to 4.09 (hay) delta units ($P < 0.05$) relative to those of the extensively managed grassland farms (see Figure 10 and Table 7). Hence, differences in N fertilizer regime obviously affected the $\delta^{15}N$ signature of all N pools on farm.

Table 7: Mean isotopic signatures of various N pools [denoted in ‰] on the study farms (A-I). Values include the standard error across both sampling periods (spring and autumn) in 2007.

Pool type	Pool description	Individual Farm								
		HNI[1)]			LNI[2)]					
		A	B	C	D	E	F	G	H	I[3)]
I	Plant biomass	4.84 ± 1.40	4.28 ± 1.20	5.98 ± 1.46	1.90 ± 1.2	1.73 ± 1.00	1.37 ± 0.90	1.86 ± 1.69	1.12 ± 1.76	1.55 ± 1.03
	Silage	4.51	4.00	6.05	2.19	1.32	1.54	0.95	2.08	2.80
	Hay	3.51	n.a.[4]	6.96	-1.13	0.42	1.09	2.94	n.a.	0.79
II	Top Soil	7.35 ± 0.48	6.06 ± 0.47	7.03 ± 0.36	4.42 ± 0.84	3.95 ± 0.41	4.66 ± 0.52	3.80 ± 0.85	3.93 ± 1.02	4.79 ± 0.50
III	Hair	6.44 ± 1.04	6.27 ± 0.48	8.35 ± 0.12	4.93 ± 0.29	4.27 ± 0.61	n.a.	4.27 ± 0.18	4.47 ± 0.12	4.63 ± 0.14
	Milk	6.37 ± 3.38	7.00 ± 2.17	n.a.	5.52	3.68 ± 0.12	n.a.	4.72	n.a.	4.32
IV	Faeces	4.58± 0.34	5.08± 0.58	6.08 ± 0.39	3.55 ± 0.81	2.55 ± 0.52	2.43 ± 1.37	3.23 ± 0.72	1.58 ± 038	2.09
	Urine	-0.25 ± 0.56	-0.08 ± 0.83	n.a.	-1.13 ± 0.92	-1.68 ± 0.75	n.a.	-1.11 ± 0.55	-1.89 ± 0.96	-2.94 ± 0.77
	Slurry	11.19	8.15	13.78	8.17	9.86	5.35	10.61	8.88	6.87
	Farmyard manure	n.a.	n.a.	n.a.	1.69	6.29	n.a.	n.a.	4.75	4.34

[1] HNI= high N input

[2] LNI= low N input

[3] On farm I all samples were only taken once (July)

[4] n.a.= not available

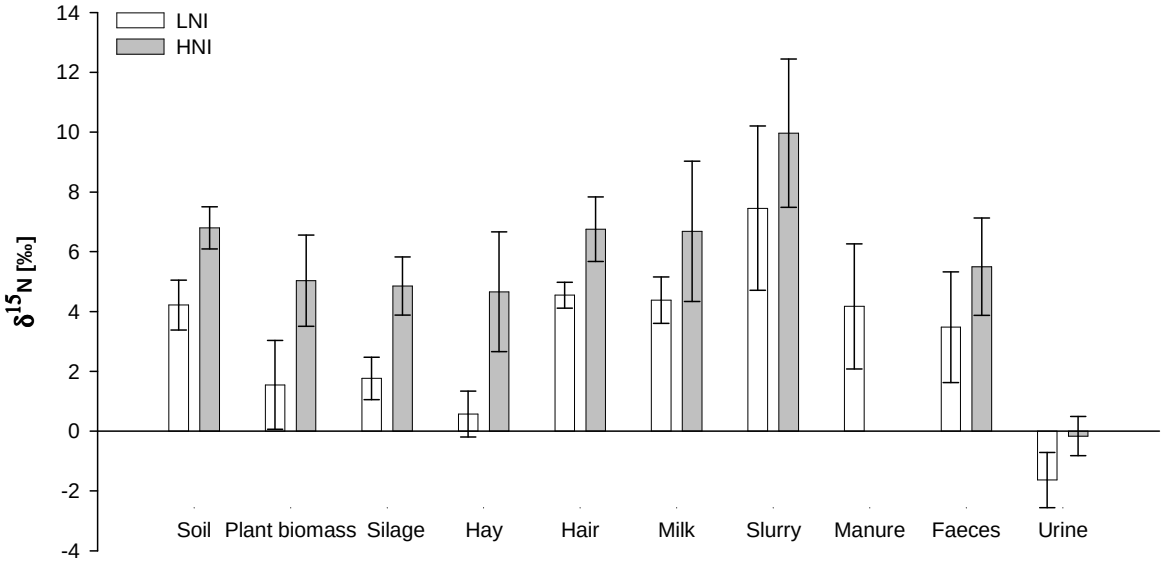

Figure 10: Mean $\delta^{15}N$ values [in ‰] of various N pools from farms based on LNI management (< 1.4 LU ha $^{-1}$) and HNI management (≥ 1.4 LU ha $^{-1}$). Error bars represent the 95 % confidence interval of the means.

For a better illustration of the $\delta^{15}N$ pattern across the different N pools, we performed a cluster analysis (Figure 11) which subdivides the preselected nine farms into three classes, HNI farms, LNI farms, and nature protection farms (NPA acreages). The latter were characterized by zero fertilizer application and therefore all measured N pools showed very low $\delta^{15}N$ values relative to the other fields (see Figure 11).

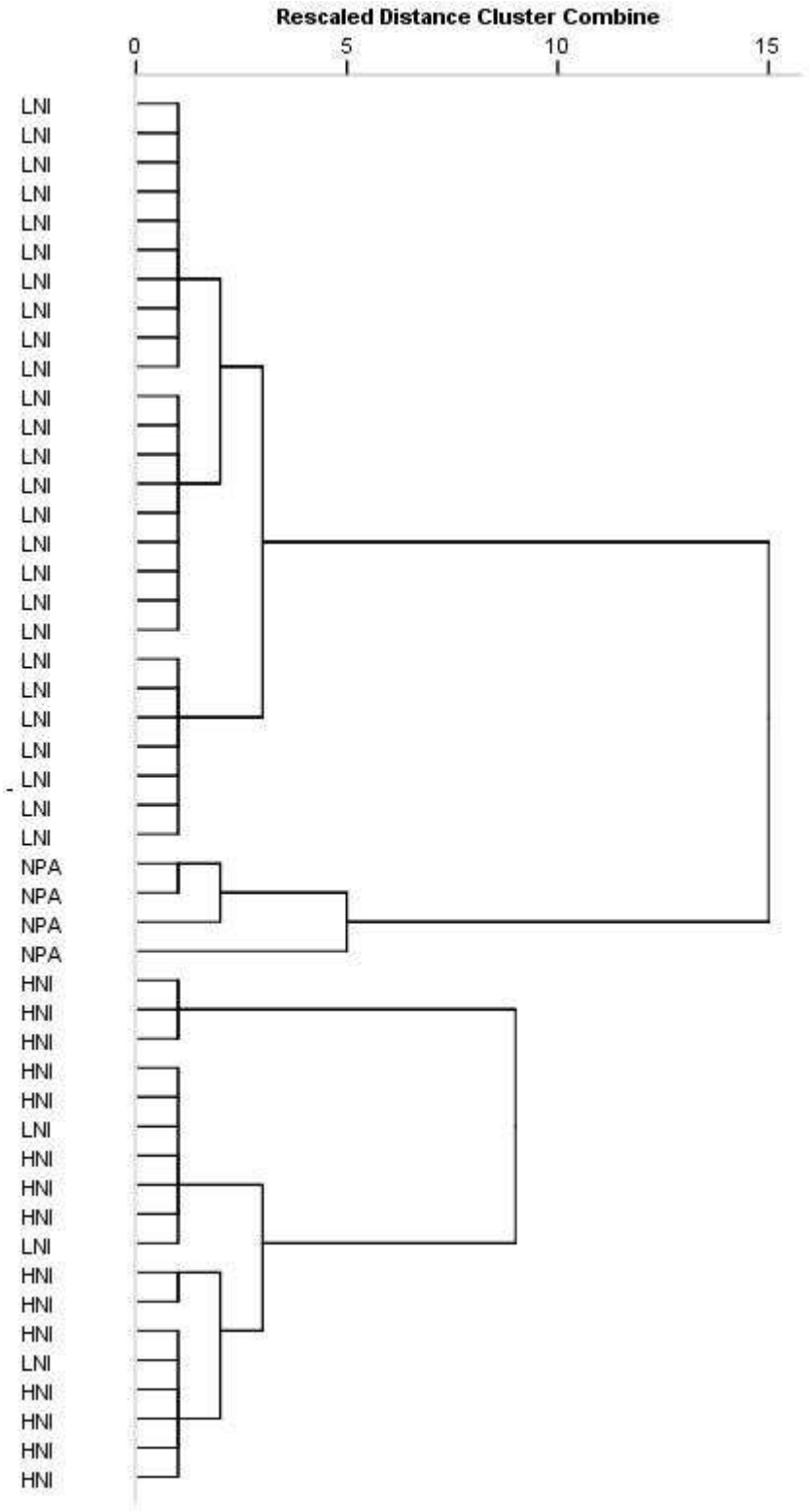

Figure 11: Hierarchical dendrogram of $\delta^{15}N$ values of topsoils and biomass obtained from the fields on all studied farms.
The fields were grouped into high N input (HNI= ≥ 1.4 LU ha^{-1} a^{-1}; farms A, B, C, in Table 1) low N input (LNI= ≤ 1.4 LU ha^{-1} a^{-1}; farms D, E, F, G, H, I in Table 1) and NPA nature protection by agreement (no fertilizers were used).

Field N balances were calculated as shown in calculated (see Table 6). On the input side, organic N was derived from animals, N output was mostly composed of harvested biomass as fresh grass, silage or hay and to a minor degree of N losses during fertilizer storage and application. Imports of chemical-synthetic N fertilizer were only found in the HNI farms and ranged from 55 to 120 kg N ha^{-1} a^{-1}. All LNI farms solely applied organic fertilizer, namely slurry and farmyard manure. The application of N fertilizers thus varied from 0 (NPA) up to 450 kg N ha^{-1} a^{-1}. N from NH_3 deposition and N_2 fixed by legumes were not included in the calculation. Feed was mostly imported as cereals and concentrates. At field scale, N-exports consisted of N removed with biomass, selling of organic N fertilizer and N losses during storage and application.

According to the N balance provided by the NRW- Chamber of Agriculture and additional notes received from the farmers, N input-output balances varied between -130 (farm F) and 196 (farm A) kg N ha^{-1} a^{-1} (Table 6).

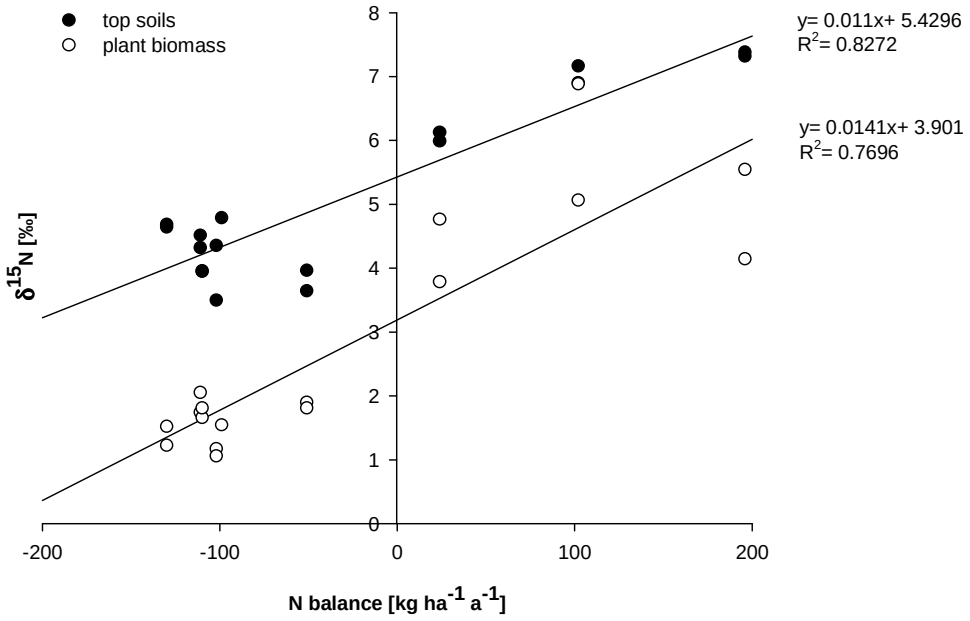

Figure 12: Mean $\delta^{15}N$ values [in ‰] of topsoils and plant biomass in relation to N balance [kg $ha^{-1}a^{-1}$], calculated as the difference of total N input (fertilizer, atmospheric deposition and symbiotic N_2 fixation) to total N output (biomass production, selling of organic N fertilizer and N losses during storage and application).

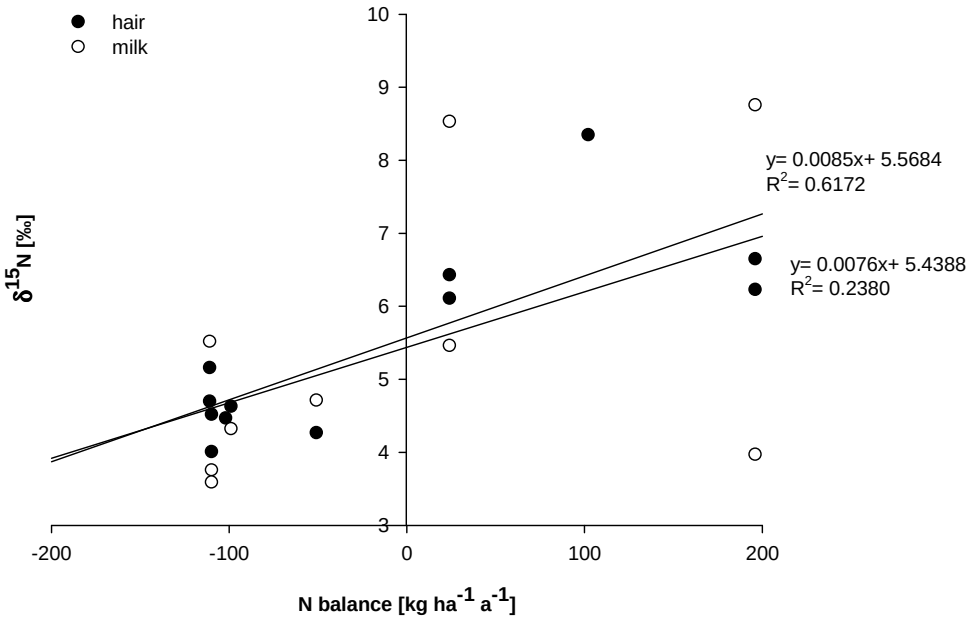

Figure 13: Mean $\delta^{15}N$ values [in ‰] of hair and milk in relation to N balance [kg $ha^{-1}a^{-1}$], calculated as the difference of total N input (fertilizer, atmospheric deposition and symbiotic N_2 fixation) to total N output (biomass production, selling of organic N fertilizer and N losses during storage and application).

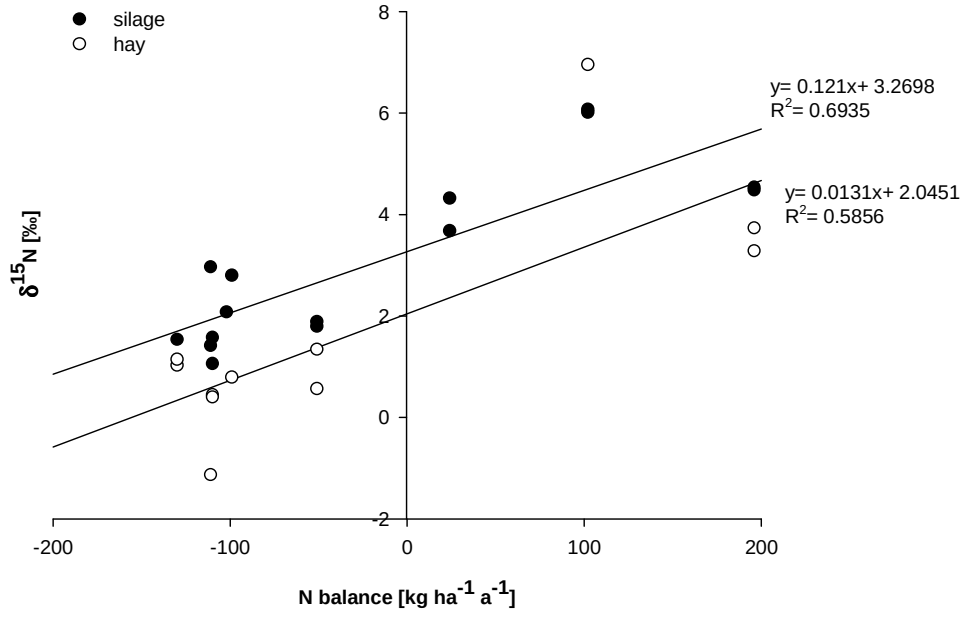

Figure 14: Mean $\delta^{15}N$ values [in ‰] of silage and hay in relation to N balance [kg $ha^{-1}a^{-1}$], calculated as the difference of total N input (fertilizer, atmospheric deposition and symbiotic N_2 fixation) to total N output (biomass production, selling of organic N fertilizer and N losses during storage and application).

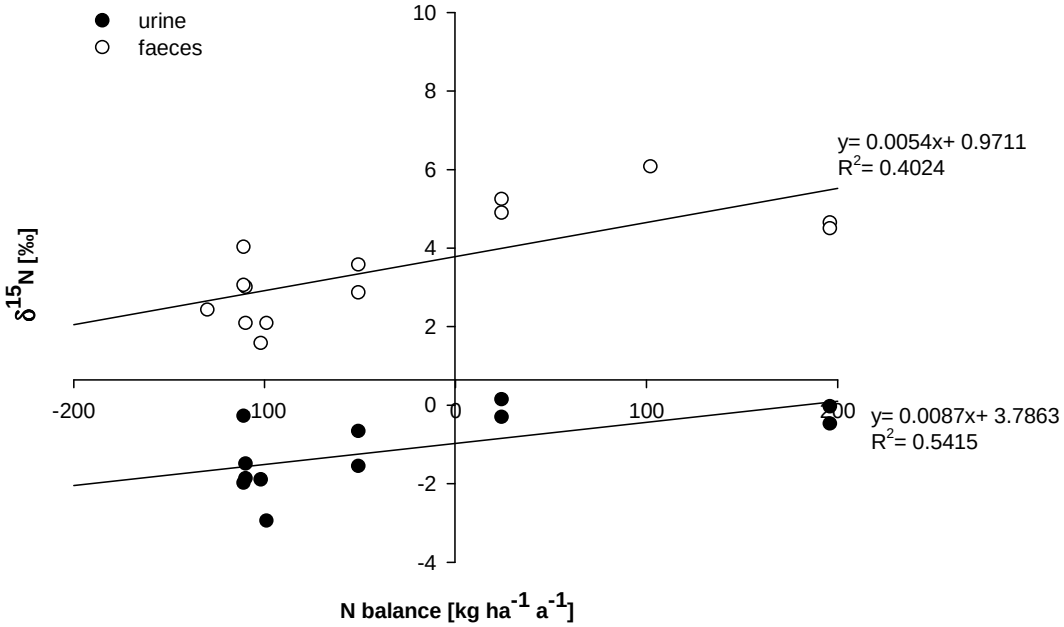

Figure 15: Mean $\delta^{15}N$ values [in ‰] of urine and faeces in relation to N balance [kg $ha^{-1}a^{-1}$], calculated as the difference of total N input (fertilizer, atmospheric deposition and symbiotic N_2 fixation) to total N output (biomass production, selling of organic N fertilizer and N losses during storage and application).

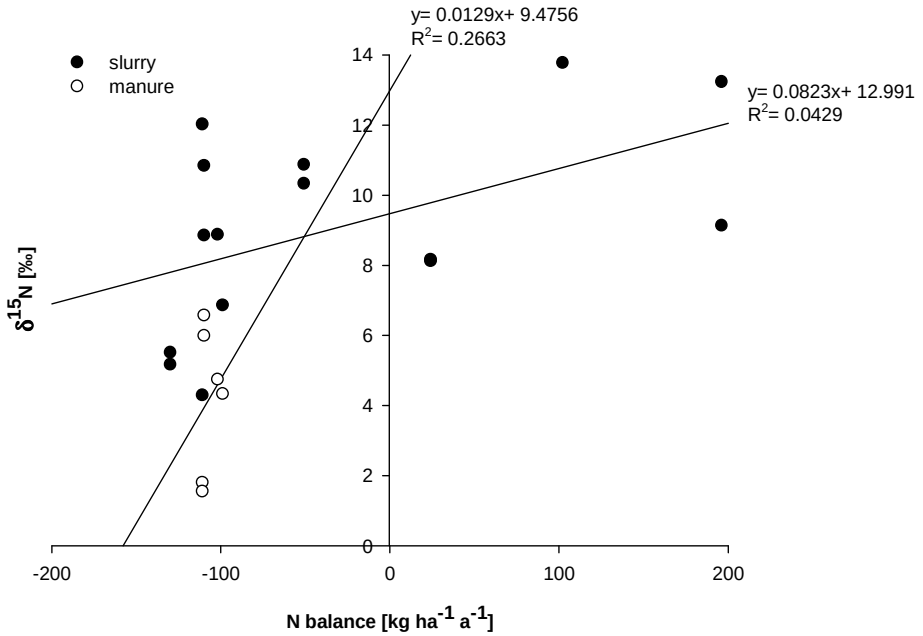

Figure 16: Mean $\delta^{15}N$ values [in ‰] of slurry and manure in relation to N balance [kg $ha^{-1}a^{-1}$], calculated as the difference of total N input (fertilizer, atmospheric deposition and symbiotic N_2 fixation) to total N output (biomass production, selling of organic N fertilizer and N losses during storage and application).

The plotting of these differences in N balances against the $\delta^{15}N$ values of topsoils indicated a general quantitative relationship. The higher the excess in the N balance was, the more positive were the respective $\delta^{15}N$ values of the topsoils (see Figure 12). Although similar close relationships were also found for $\delta^{15}N$ values of plant biomass and silage, no such clear relationships were found for the remaining N pools (hair r^2= 0.61; hay: r^2= 0.59; urine: r^2= 0.54; faeces r^2= 0.40; slurry: r^2= 0.27; milk: r^2= 0.24 and manure: r^2= 0.03; see Figure 13 to Figure 16). Apparently, some N pools such as soil, plant biomass and silage may be suitable indicators for reflecting the N balance.

4.3.2 Impact of stocking rate and total milk production on $\delta^{15}N$ values

Since mean $\delta^{15}N$ values of various N pools correlated strongly with the N intensity on farm, we presumed that they could also be used to differentiate between other characteristics of low and high N input management, such as stocking rate (in livestock unit = LU ha^{-1}) or total milk production on farm (t a^{-1}). In Figure 17 the $\delta^{15}N$ signatures of miscellaneous N pools are plotted against the average stocking rate on farm. The highest correlations were observed with soil, biomass, silage, hay, and hair.

Hence, particularly those N pools that integrate information over longer periods of time like soil and hair were very suitable to reflect the previous animal husbandry practices. Since the $\delta^{15}N$ of biomass was strongly correlated to the $\delta^{15}N$ of soil, there was also a strong relationship between the $\delta^{15}N$ of biomass and the stocking rate on farm. Thus, even the above ground biomass retains information that is valid only for the duration of the current growing season. The same trend and correlation was observed for silage and hay. Hence the $\delta^{15}N$ of biomass (including silage and hay) served as an indicator for stocking rate that was as good as the $\delta^{15}N$ of long-term N pools for total N balance. In contrast, the N pools that appear on farm for comparatively short-term periods of time, like excreta and milk, performed much lower r^2- values. Comparable results were found when plotting the $\delta^{15}N$ signatures against milk production (Figure 18).

High stocking rates and high total milk production were accompanied by high N inputs and thus reflected a positive N balance. HNI farms produced considerable N surplus (24 to 196 kg N ha^{-1} a^{-1}), whereas all LNI farms incurred N deficits (-130 to -51 kg N ha^{-1} a^{-1}). Hence, correlations of the $\delta^{15}N$ signature of N pools to the stocking

rate contributed to their overall relationship to N balance as shown before in Figure 12.

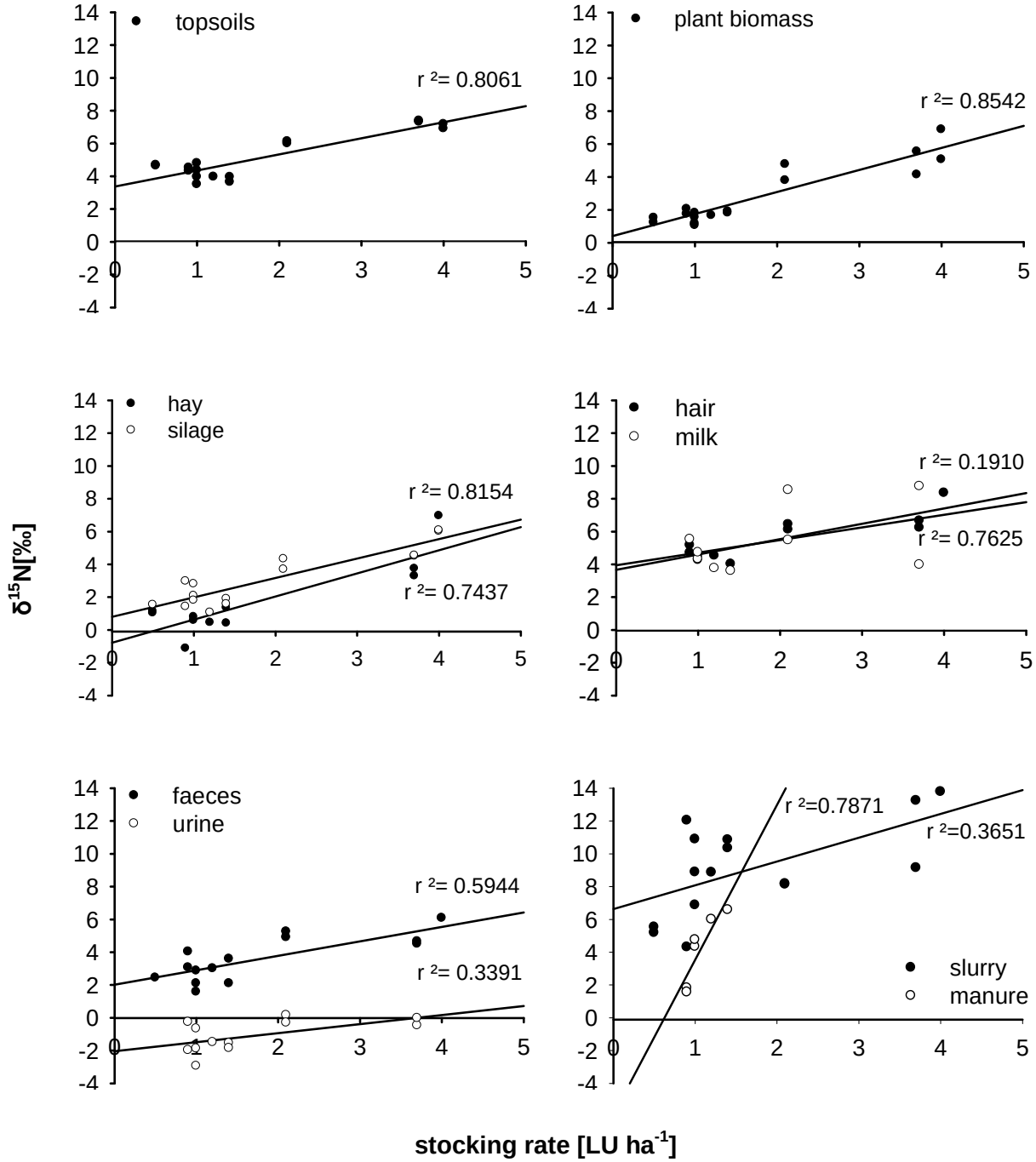

Figure 17: Mean $\delta^{15}N$ values [in ‰] of various N pools, grouped in different N-intensity systems (grassland farms based on low or high N input management) as related to the stocking rate [LU ha^{-1}].
Samples were taken twice in April/May and October/November in 2007, except for farm I, for which only one sampling was carried out in July 2007.

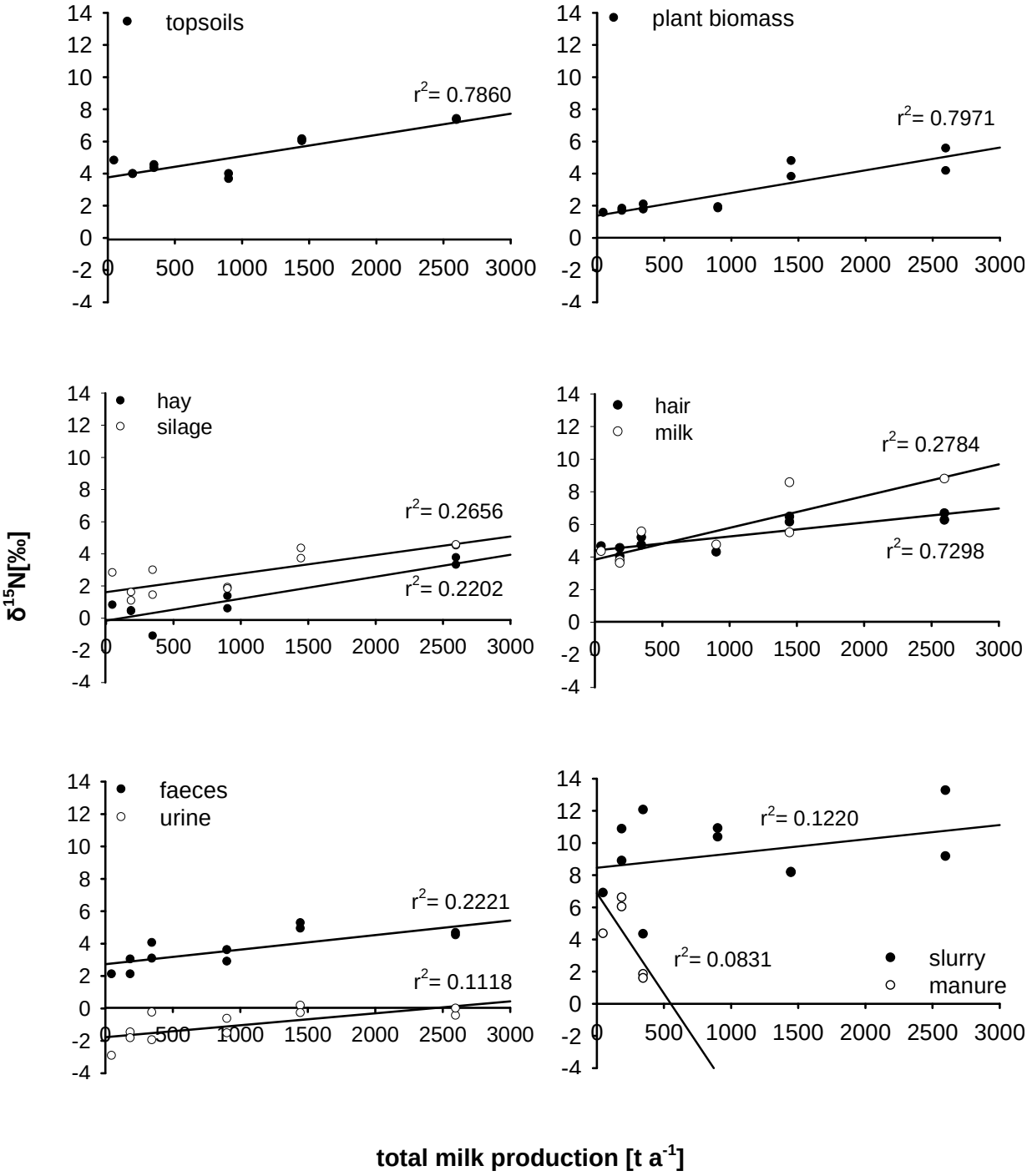

Figure 18: Mean $\delta^{15}N$ values [in ‰] of various N pools, grouped in different N-intensity systems (grassland farms based on low or high N input management) as related to the milk production [t a^{-1}].
Samples were taken twice in April/May and October/November in 2007, except for farm I, for which only one sampling was carried out in July 2007.

4.3.3 $\delta^{15}N$ values of plant biomass and soil as influenced by fertilizer application

Higher productivity on farm frequently goes along with higher N input. We hypothesized that the isotopic signature of the $\delta^{15}N$ pools is generally influenced by the amount of applied N and application technique as well as by the distance between farmyard and pastures/meadows. This should hold especially for those N pools that integrate over a longer residence time, like the topsoil layer. When we relate the amount of fertilizer input to the $\delta^{15}N$ values of topsoils and plant biomass, we could confirm a significant positive relationship between both (Figure 19, topsoils $r^2 = 0.81$, plant biomass $r^2 = 0.71$).

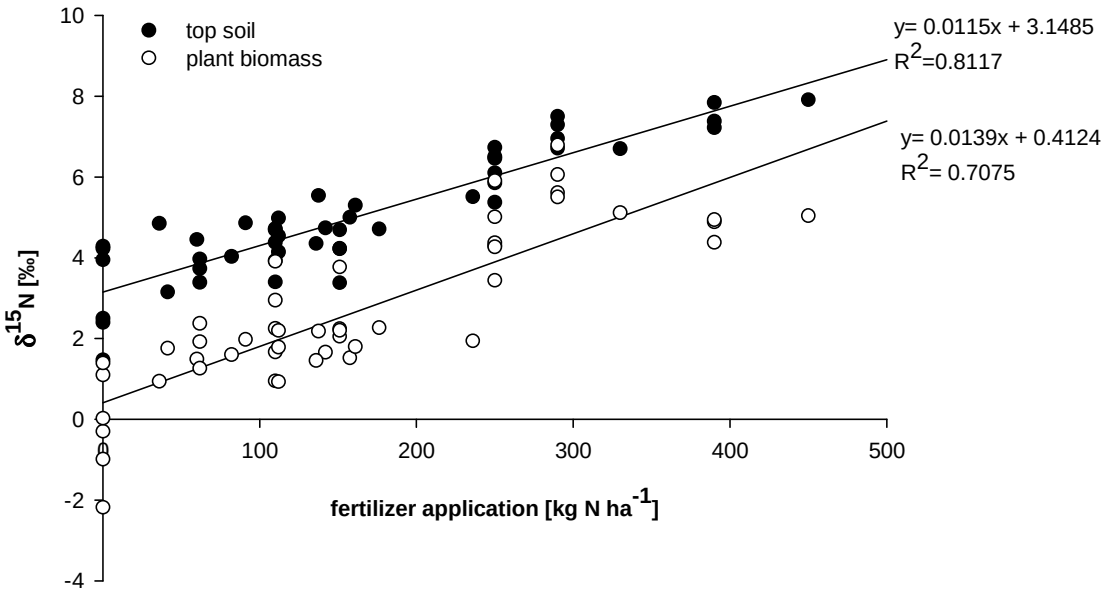

Figure 19: Mean $\delta^{15}N$ values of topsoils (filled dots) and plant biomass (open dots) in relation to organic and mineral N fertilizer input [kg N ha^{-1}].
For NPA fields, no fertilizers were applied.Each sampling data point refers to the mean value of five samples taken on each of the five probed fields of the respective farm, except for farm H, where eight fields were considered.

The correlation between the $\delta^{15}N$ values of topsoils and plant biomass on the one hand and fertilizer input on the other was lower when estimated amounts of N fixed by legumes were included into the calculation (see Figure 20, plant biomass $r^2 = 0.64$; top soil $r^2 = 0.77$). Mean $\delta^{15}N$ values of plant biomass were lower than those of topsois (Figure 19) because plants discriminated against the heavier ^{15}N isotope by

2.5 delta units, on the average (Kriszan et al., 2009; Watzka et al., 2005; Wrage et al., 2011). In NPA farms even negative $\delta^{15}N$ values were recorded.

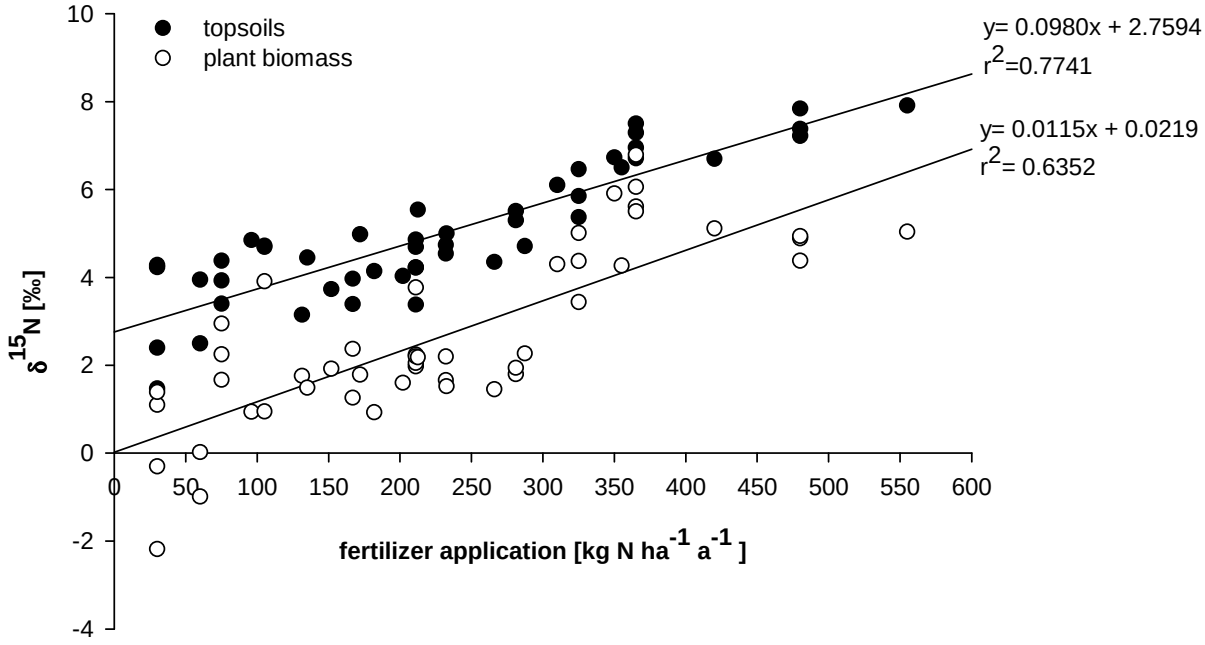

Figure 20: Mean $\delta^{15}N$ values of topsoils (filled dots) and plant biomass (open dots) in relation N input [kg N ha^{-1}] by organic and mineral fertilizer and estimated fixed N by legumes and atmospheric N.
Each sampling data point refers to the mean value of five samples taken on each of the five probed fields of the respective farm, except for farm H, where eight fields were considered.

4.3.4 Spatial patterns and the impact of the application technique

Frequently, fertilizers are not evenly distributed on farms mainly because larger amounts of fertilizer are applied near the stables. Such a irregular spatial distribution is as important for fertilizer N use efficiency as is the application technique. To account for such phenonema, we grouped our biomass and soil samples into three distance categories, i.e. close (average distance = 100 m), intermediate (average distance = 900 m), and far (average distance = 2200 m) away from the centre of major farming activity, *i.e.* cowshed, barnyard, slurry container and feed store. No significant difference between the three groups of sampling regions could be determined within each HNI farms. On HNI farms, no significant difference between the three distance categories could be determined. In contrast, LNI farms showed significantly higher $\delta^{15}N$ values in samples taken close to the farms than in those

taken further away, and this was found for both the top soils and the plant biomass (Figure 21).

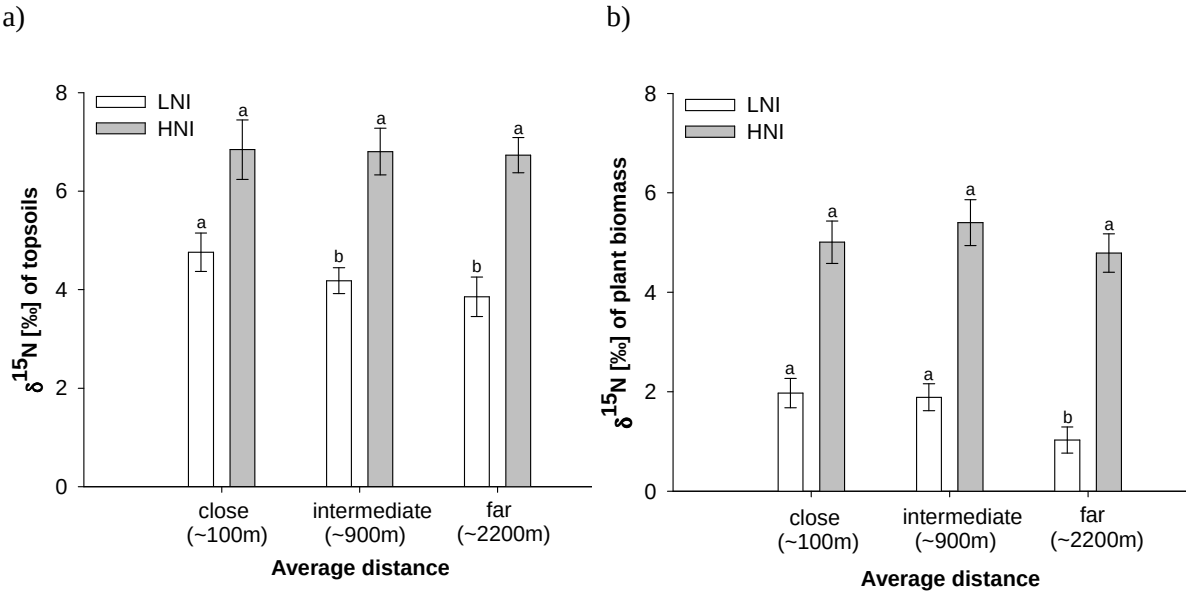

Figure 21: Mean $\delta^{15}N$ values in [‰] of a) topsoils and b) plant biomass of grassland farms based on low N inputmanagement and high N input management at sites of different distances to the farmyard.
Values with the same letters refer to no significant differences (one-way ANOVA, $P < 0.05$). Error bars represent the 95 % confidence interval of the means.

We excluded the NPA fields from the calculation since they were not fertilized at all. Moreover they were not present on HNI farms. The fertilizer application technique did not leave a precise mark concerning the in $\delta^{15}N$ values of topsoils and plant biomass (Figure 22a, b). Accordingly there were only small but significant differences within low-emission and high-emission fertilizer application, with even higher values in the topsoils of the LNI farms with high emission application. With increasing amount of N losses during spreading, the $\delta^{15}N$ of topsoils increased significantly within the LNI farms. Analysing the plant biomass, only the NPA acreages of LNI farms showed lower $\delta^{15}N$ values.

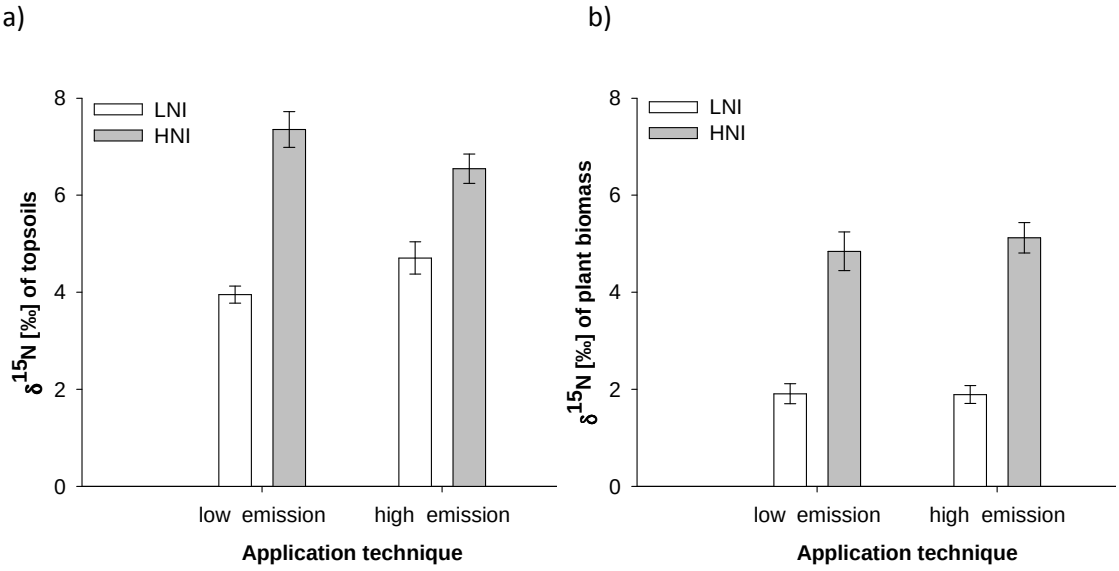

Figure 22: Mean $\delta^{15}N$ values in [‰] of a) topsoils and b) plant biomass of grassland farms based on HNI and LNI management as related to the N fertilizer application techniques (low and high emission application).

4.4 Discussion

In this study, isotopic signatures of biomass and soil closely reflected the underlying N management on farms. Main differences between the isotopic signatures of various N pools were affected by considerable input of N from organic manure, causing N volatilisation and ^{15}N enrichment. In contrast, mineral N derived from atmospheric di-nitrogen through Haber-Bosch technology exhibited lowest $\delta^{15}N$ values. Hence, $\delta^{15}N$ values of about -1 ‰ are common for mineral N-fertilizers like calcium ammonium nitrate (Shearer et al., 1974; Wassenaar, 1995; Kriszan et al., 2009), which have only a small if any effect on the $\delta^{15}N$ signature of the N pools sampled here.

Organic fertilizer like cattle slurry or farmyard manure are constantly enriched in ^{15}N, due to fractionation processes during digestion of forage and manure storage (Jarvis and Kerley, 1996; Choi et al., 2002), ranging from 6-13 ‰ in earlier reports (Wassenaar, 1995; Glaser et al., 2001; Choi et al., 2002; Bol et al., 2005; Watzka et al., 2006). In this investigation, the $\delta^{15}N$ isotopic signatures of all sampled organic fertilizers were in the same magnitude as reported elsewhere (Wassenaar 1995; Choi, 2002; Bol et al., 2005). The processes accounting for the high $\delta^{15}N$ fractionation rates mainly comprise ammonia volatilization (0 - 29 ‰), nitrification (0 - 35 ‰) and denitrification (0 - 33 ‰) (Handley and Raven, 1992; Högberg, 1997), which all permanently occur on farms.

With higher N input to the system the $\delta^{15}N$ values of both biomass and soil increased significantly, as was found already earlier in controlled experiments (e.g. Yoneyama 1996, Choi et al., 2003, Bol et al., 2005, Kriszan et al., 2009). The amount and the type of fertilizer influenced the N losses and thus the $\delta^{15}N$ values (Watzka et al., 2006, Kriszan et al., 2009). There was even a positive linear relation between the intensity of N application in grassland farms and the $\delta^{15}N$ values of plant biomass and topsoils (Figure 19), suggesting that similar processes accounted for the N losses and changes in farm $\delta^{15}N$ values across all sites. Only the scale of these changes varied due to different degree of N losses. In this respect, HNI farms could be clearly separated from LNI and NPA farms in the cluster analysis.

High stocking rates, a major indicator for high N input managed grassland farms, may lead to high accumulation of organic N fertilizer (Schwertl et al., 2005). The higher the stocking rate, however, the higher is the risk of N losses, presumably through NH_3 volatilization (Högberg, 1995). Thus, HNI farms with a stocking rate above 1.4 LU ha^{-1} were characterized by higher amounts of organic N per area, resulting in increased $\delta^{15}N$ of plant biomass and topsoils and increasing losses of N from the slurry or farmyard manure. According to our findings the $\delta^{15}N$ signature of different farm N pools are thus plausible indicators of the efficiency of N management. On the contrary, Wrage et al., (2011) did not observe an effect of stocking rate on the ^{15}N signature of N pools such as soil, plant biomass, hair and wool, but both studies had a relative short runtime of only three years. Furthermore, the N input in the study of Wrage et al., (2011) was considerable low ranging from 19.0 to 39.1 kg N ha^{-1} a^{-1}, but the stocking rates varied between 1.1 and 3.1 LU ha^{-1} a^{-1}. Nonetheless, according to our findings, the $\delta^{15}N$ signatures of different farm N pools indeed indicate the efficiency of the underlying N management.

LNI farms did not cause N surpluses, however, especially on pastures notable N losses have been unavoidable, as grazing animals usually excrete 75-90 % of feed intake on spots (Ball et al., 1979). The amount of N returned to the pastures in these spots is naturally high (Wachendorf et al., 2005) and not evenly distributed. High N losses due to nitrate leaching or volatilisation take place particularly under these urine and dung patches (Stout et al., 1996; Wachendorf et al., 2005; Wrage et. al., 2011). Consequently, in this study also the LNI farms exhibited positive $\delta^{15}N$ values though to at smaller extent than the HNI farms.

Several field plots of the LNI farms, mainly the pastures/meadows close to the farmyard, did not exhibit the expected low $\delta^{15}N$ values. This was probably caused by

high local stocking rates and long grazing periods especially on the paddocks in vicinity of the barn. Moreover these plots were also used for the first cut. That way they likely received more organic fertilizer than meadows located farther away from the farm or at higher altitude with less favourable conditions for animal husbandry. In all of the investigated grassland farms we found the highest $\delta^{15}N$ values of topsoils and plant biomass on pastures and grassland located close to the farmyards.

For instance the Austrian farm (farm I) showed a very inhomogeneous spatial distribution of N input. It was characterised by an organically farming system with a very close nitrogen circuit, with low amounts of N loads as concentrate fodder and no mineral fertilizer input. In contrast to the fields that are difficult to cultivate due to poor soils or high altidude, the soils or plant biomass of pastures close to the farm showed high $\delta^{15}N$ values. Despite the Austrian farm had a negative N balance, only a marginal difference of the ^{15}N signatures was observed on the pastures close to the farm compared to ^{15}N signatures typically found in tops soils and plant biomass of conventional farms with positive N balances.

Topsoils and plant biomass of fields that were located far away from the farms showed lower $\delta^{15}N$ values (Figure 21a, b). This situation is plausible, since large distances from the farmyard to the field result in higher costs for the farmers, due to higher expenditure of time and fuel consumption. As a result, fields dislodged from the farm yard are frequently neglected with respect to fertilizer application, resulting also in lower overall N losses and less raised $\delta^{15}N$ values (Figure 19 and Figure 20).

In general, NPA acreages that did not receive any fertilizer due to the governmental requirements were located at the greatest distance to the farmhouses. And indeed, we found the lowest ^{15}N signatures within the topsoils and plant biomass of the NPA plots (Figure 19 and Figure 20).

The effects were most pronounced on the LNI farms, the $\delta^{15}N$ values of which likely responded more sensitively to increased fertilizer input nearby the stable. On LNI farms, this finding supported thus our hypothesis that farmers tend to fertilize grassland most intensively when it is located in the vicinity of their stables, and that N load generally tends to decrease with increasing distance from that place. The high $\delta^{15}N$ values reveal potentially over-fertilization on single pastures or meadows even if the farm management is subject to special regulations limiting the stocking rate and therefore the amount of incoming organic fertilizer.

No such clear differentiation could be made for the HNI farms, since animals were kept indoor all- the-year. Nevertheless, our data clearly show that it is crucial to

account not only for the N- intensity of grassland management but also for the distribution of fertilizer application within the farm area.

Effects of the fertilizer technique on soil $\delta^{15}N$ values were only indicated for the LNI but not for the HNI farms (Figure 22). However, compared with the spatial distribution of soil and plant $\delta^{15}N$ values, these effects were rather small. On the one hand these findings may indicate that major N losses occurred also from soil after application of the fertilizer, irrespectively by which technique. On the other hand it may indicate that the use of modern application techniques on soil $\delta^{15}N$ values covers a shorter period of time than the effect of the amount and type of fertilizer N input and the grazing system. Hence, elucidating the rates of soil and plant $\delta^{15}N$ changes now warrant further attention.

4.5 Conclusions

The data supports the hypothesis that high N surplus in grassland farms that extends periods of time can be traced back by elevated $\delta^{15}N$ values of soil and biomass, and, to a smaller extend, also by the hair of the animals. The higher the $\delta^{15}N$ value, the lower the overall farm N efficiency of the total N applied. Among the different parameters and N pools tested, strong correlations between the N balances and the $\delta^{15}N$ values of topsoils and plant biomass were found and furthermore between the stocking rates and the $\delta^{15}N$ values of topsoils, plant biomass, silage, hay and hair. All these N pools that integrate over several months to seasons. In other words, the longer the pool remained in the system, the higher the accuracy of using its $\delta^{15}N$ value as an indicator for N efficiency on a total farm scale. Similarly, the longer the N compounds are recycled in the system, the stronger is the N isotope signature influenced by discrimination processes, and the better is the information that can be depicted from these isotopic measurements about N input and output ratios. Especially when farming practice is not well recorded, screening for small $\delta^{15}N$ values in soil, plants, silage and hair may help to identify good farming practice.

5 Summary

Nitrogen is one of the most important elements in agicultural production. It plays an important role as fertilizer, in atmospheric deposition and biological fixation. In animal husbandry, a considerable amount of nitrogen taken up by the fodder is recirculated to the soil as excreta. However, nutrient losses due to these processes are inevitable, and thus nutrient losses should be avoided as much as possible, with respect to both ecologically and economically motives.

Modern biological investigations apply stable isotopes for the exploration of nutrient fluxes in ecosystems, based on the principles of the different physical properties of isotopes. During turnover processes, the light isotope will be preferably converted into the respective products compared to their heavier analogous. Hence, the lighter ^{14}N isotopes are found in the consumed forms of nitrogen and an enrichment of the heavier ^{15}N isotope is observed in the remaining substrate. The range of this fractionation depends mainly on the amount of nitrogen losses.

Motivated by promising applications of stable isotopes in agriculture, the aim of this work was to determine whether $\delta^{15}N$ signatures of different N pools reflect the underlying long-tem N management. Furthermore, it was tested if $\delta^{15}N$ signatures of various N pools are suitable sustainability indicators of farm management and if they allow a retrospective estimation of effiency of N fertilizer use on grassland farms.

To this end, samples were taken on two long-term experimental grassland plots located at the University Bonn Grassland Research Station in Rengen, Germany, which were constantly fertilized with N and cut in a time period of 20 and 22 years. In a final study, eight grassland farms in North Rhine-Westphalia and Rhineland-Palatinate, Germany and one farm in Styra, Austria were investigated which differed in N management .

Samples of various N pools, namely soil, plant biomass (divided in total plant biomass and four dominating species (*Lolium perenne* L.*, Festuca rubra* L.*, Taraxacum officinal Web. and Trifolium repens / pratense* L.)*,* hay, silage, milk, hair, urine, faeces, slurry and manure were considered. The $^{15}N/^{14}N$ isotope ratios and the total N content of the soil samples were determined by dry combustion in an elemental analyser.

Do the $\delta^{15}N$ values of soil and biomass reflect the mineral N fertilizer management?

Within the first field-site experiment on plot scale with two N management systems differing in the cutting frequency and the N input, $\delta^{15}N$ signatures of soil and plant biomass were determined. The mean $\delta^{15}N$ values of the above ground plant biomass were lower than that of the respective topsoils. The specific plant $\delta^{15}N$ values could be attributed to differences in plant growth, resource acquisition strategies or $\delta^{15}N$ values of the soil N pools for which they compete. However, no significant differences in the $\delta^{15}N$ values of plant biomass with increased N fertilization were observed within the two-cut system at low N input. Only a tendency towards higher $\delta^{15}N$ values for increased amounts of N fertilizers could be observed in the more intensive four cut system, but differences were not significant.

Higher soil $\delta^{15}N$ signatures were observed at increased depths, but they did not reflect any significant differences between the cutting and N input as was similarly found for plant biomass. Hence, the ^{15}N signature of plant biomass and soil could not be related to possible N losses associated with N application rates up to 240 kg N ha^{-1} a^{-1}. Thus, they are not suitable as indicator for different N input, at least when chemical-synthetic fertilizers were used exclusively. This may result from two issues, 1) the $\delta^{15}N$ signature of chemical synthetic fertilizer is close to zero permil since the contained N originates from atmospheric nitrogen, and 2) there was only a small extent of N losses, which cause highly ^{15}N enriched products that is reflected by higher $\delta^{15}N$ values of plant biomass and soil.

Is it possible to indicate the N efficiency after long-term fertilization (mineral and organic) by analysing the $\delta^{15}N$ values of soil and biomass?

In the second experiment the investigation was extended to organic N fertilizer with an amount of up to 480 kg N per year. In contrary to the first experiment, all plots of the field lysimeter experiment were cut equally four times a year. Analogous to the first experiment, plant and soil were sampled and the corresponding $\delta^{15}N$ signatures were determined.

In contrast to the first experiment, for which a chemical synthetic N fertilizer was applied only, significant differences between plots treated with different amounts and types of N fertilizers were obtained. The results confirm those of Watzka et al. (2006), indicating that long-term fertilizer application has a significant effect on the $\delta^{15}N$

values of both the soil and plant biomass, specifically with increasing amounts of N fertilizers. Especially organic N fertilization resulted in siginificantly higher $\delta^{15}N$ values of the soil and plant biomass.

In order to exclude that the $\delta^{15}N$ value of the organic N fertilizer itself caused the higher $\delta^{15}N$ values of the soil and in the plant, a theoretical $\delta^{15}N$ value was calculated that consists of both the original soil $\delta^{15}N$ value plus the $\delta^{15}N$, which would arise from fertilization. This theoretical $\delta^{15}N$ signatures recovered in the SOM were subsequently compared to the measured ones. Since some residual fertilized N always remains in the soil (Jenkinson et al., 2004), I concluded that N discrimination processes occur within the organic fertilizer N, and hence the heavier ^{15}N- isotopes remain in the soil. As it is obvious from the results presented in Chapter 3, the differences between measured $\delta^{15}N$ and calculated $\delta^{15}N$ values were positive in each treatment, with the highest fractionation values arising from the highest N input treatment, thus indicating that ^{15}N fractionation occurs before or during the incorporation of the fertilizer N into the soil.

Additionally, the soil samples were separated into two different SOM fractions to trace back the pathway of fertilizer ^{15}N into stable SOM pools in order to verify that not the signal of the fertilizer itself was responsible for increasing $\delta^{15}N$ values, but the N losses during the turnover processes. Moreover, the scope was to investigate if $\delta^{15}N$ values can be used for an estimation of the type and development of N losses over time.

The increase of $\delta^{15}N$ signatures in the order plant biomass < coarse sand sized SOM < fine sand sized SOM < bulk soil reflects the increased $\delta^{15}N$ isotope fractionation during soil organic N genesis. Along with increasing amounts of fertilizer N, increasing $\delta^{15}N$ values in both SOM fractions were observed, suggesting that a significant part of the added N already affected SOM pools such as fine SOM. The turnover time of this SOM pool was estimated to a period of approximately < 15 years (arable soil; Balesdent and Mariotti 1996). Since no ploughing took place on the grassland plots for a long period, the turnover time may be even longer. Hence, it was not the short but the long-term cycling of fertilizer N that affected soil $\delta^{15}N$ values. However, it was not possible to narrow down the time more precisly. Evans (2001) and Robinson (2001) assumed that the ^{15}N isotope technique might not be applicable to quantify surplus situations on grassland fields or farms. This may remain true for short-term fertilization trials. The data obtained in the present study,

however, clearly show that application of excess N over a long time period is detectable using natural $\delta^{15}N$ abundance measurements.
The ^{15}N signatures of the SOM in long-term treatments provide some kind of a fingerprint of the underlying N fertilization, whereas plant specific $\delta^{15}N$ values are more reliable with respect to short-term variations.

Is it possible to reveal the underlying N management on grassland farms using the natural abundance of ^{15}N of various N pools?

Based on the results and conclusions documented in Chapter 2 and 3, the study was extended on nine common grassland farms to assess the use of $\delta^{15}N$ signatures as indicator for N management on grassland farms in practise.
The classification into low and high N input management was based on the indicators such as stocking rate (LU ha^{-1}), total milk production (t a^{-1}) and the N fertilizer input (kg^{-1} a^{-1}).
The assessed data support the hypothesis that high N surplus in grassland farms over extended time periods can be traced back by enhanced $\delta^{15}N$ values of soil and biomass, but also in the hair of the animals. Especially high stocking rates, which are a major indicator for high N input managed grassland farms, are responsible for high accumulation of organic N fertilizer (Schwertl et al., 2005) with inevitably high N losses caused mainly by NH_3 volatilization (Högberg, 1995). Thus, HNI farms with a stocking rate above 1.4 LU ha^{-1} are characterized by higher amounts of organic N per area, resulting in increased $\delta^{15}N$ values of plant biomass and topsoils together with increasing losses of N from the slurry or farmyard manure.
No significant difference concerning spatial patterns and the application techniques could be determined within the HNI farms. However, the $\delta^{15}N$ values observed for HNI were significantly higher than that of the LNI farms.
LNI farms, however, showed significantly higher $\delta^{15}N$ values in samples taken close to the feed stocks for both topsoils and plant biomass due to the higher N losses during grazing.
Concerning the impact of the application technique, small but significant differences within low-emission and high-emission fertilizer application in topsoils of LNI managed farms were achieved. Analysing the plant biomass, only the NPA plots of LNI managed farms showed lower $\delta^{15}N$ values.

Contrary to the hypothesis, I observed even larger $\delta^{15}N$ values in HNI farms that make use of modern low-emission fertilizer application techniques rather than outdated application techniques. Obviously, the influence of very high amounts of organic and mineral fertilizer usage on the isotopic discrimination is much more signifincant than other side affects such as the distance to the farm.

Even though the ^{15}N signature is a useful indicator for detecting the use of organic nitrogen retrospectively, it has to be taken into account that in practice, several parameters have an impact on the ^{15}N signature of the given N pool:

- Water regime and soil type: stagnant water regime in combination with loamy or silty soils may lead to higher $\delta^{15}N$ signatures due to increasing N losses through denitrification.
- Only high fertilization with organic manure could be recovered with the increased $\delta^{15}N$ signature, whereas overdoses of mineral fertilizer could not be detected due to the $\delta^{15}N$ signature close to zero.
- Previous long-term N mangement may affect the $\delta^{15}N$ signature significantly. Hence, single fertilizer applications or short-term changes of the N mangement may not be detected properly. A change of the N mangament from low N input to high N input and vice versa, as it is often found in NPA fields will be only measurable after long time periods.
- Ecological farming: generally, ecological farming is characterized by LNI management, and hence also by low $\delta^{15}N$ values due to the low stocking rate (< 1.4 LU ha^{-1}). However, increased $\delta^{15}N$ values similar to the conventionell farming based on higher stocking rate and HNI mangement could also be found in these cases (e.g. Farm I, Austria). These observations suggest that despite lower stocking rates the spatial distribution of fertilizer input may also affect the usefulness of isotopic abundances of ^{15}N for tracing N efficiency at these farms. Grassland located close to the stable may receive more organic fertilizer due to pasturing and additional manure or slurry application in surplus.

Hence, the $\delta^{15}N$ signature of soil, plants and animal tissue and products is only useful as a crude indicator and is not capable to identify the precise amount of nitrogen used. Due to the fact that there are regions with strongly varying intensities of the N fertilization, a classification or mapping of $\delta^{15}N$ signature of e.g topsoils distributed over Germany or Europe would be instructive and of interest for better control of the

N management. With the help of a $\delta^{15}N$ signature database, it may be possible to identify farms or single fields with excessive N fertitilizing in short time. Selective samples may yield informations about exceeding the limit of (organic) fertilization regulated by law. It is almost impossible to consider the exact amount of fertilizer input, but high $\delta^{15}N$ signatures reflect fertilizer overdoses over a long-term period. Consequently, further assessements with higher sample sizes are needed to get a more comprehensive overview. However, $\delta^{15}N$ signatures may help to identify these areas, and thus they provide a helpful tool for farmers to change and impove the N mangement in order to avoid negative ecological and economical N losses. Specifically in case of a well recorded farming practice, screening for low $\delta^{15}N$ values in soil, plants, silage and hair can be useful to verify good farming practice.

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VII. List of Publications

Peer- reviewed journals:

Kriszan, M., Amelung W., Schellberg J., Gebbing T., Kühbauch W. (2009). Long-term changes of the $\delta^{15}N$ natural abundance of plants and soil in a temperate grassland. *Plant and Soil* 325: 157- 169.

Kriszan M., Schellberg J., Amelung W., Gebbing T., Pötsch E., Kühbauch W. (2012). Revealing N management intensity on grassland farms based on natural $\delta^{15}N$ abundance. *Agriculture, Ecosystems and Environment* [under review].

Conference proceedings:

Kriszan, M., Kühbauch W., Amelung W., Schellberg J., Gebbing T. (2008). Long-term changes of ^{15}N natural abundance of plants and soil in grassland. *Grassland Science in Europe* 13: 601- 603.

Kriszan, M., Kühbauch W., Amelung W., Schellberg J., Gebbing T. (2007). Effect of long-term nitrogenous losses of the ^{15}N natural abundance. *Mitt. AG Grünland und Futterbau* 8: 221- 225.

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