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Nitrogen-15 natural abundance is robust to quantify nitrogen transfer from clover to grass in temporary grassland

Andreas Hammelehle [a,1], Jochen Mayer [a], Andreas Lüscher [b], Astrid Oberson [c,*]

[a] Agroscope, Water Protection and Substance Flows, 8046, Zurich, Switzerland
[b] Agroscope, Forage Production and Grassland Systems, 8046, Zurich, Switzerland
[c] ETH Zurich, Institute of Agricultural Sciences, Group of Plant Nutrition, 8315, Lindau, Switzerland

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ABSTRACT

Nitrogen (N) transfer from clover to grass may present a significant input of symbiotically fixed clover N in grasslands. We determined grass N transferred from clover over two years using both ^{15}N natural abundance (NA) and ^{15}N labelling (LAB) methods. These methods have not previously been compared under identical environmental and management conditions. We established a model clover-grass mixture in a field experiment that had been fertilised with no, organic, or mineral N for three decades. With the NA method we used procedures differing in proxies that either represented the δ^{15}N of the N sources clover N and plant available soil N while LAB procedures differed in labelling strategies and/or calculation. Different proxies in NA had little impact on the proportion of grass N transferred from clover. The δ^{15}N of the two N sources differed significantly under all fertilisation treatments, by 4.0‰–5.9‰ under zero and organic fertilisation, though often less than 2‰ under mineral fertilisation. The by-treatment average proportion of N transferred from clover ranged from 27% to 55% of grass N for all NA procedures, and from 21% to 68% for all LAB procedures. The LAB procedures were affected by temporally non-uniform ^{15}N enrichment of clover roots. For both methods, all treatments, and both years, about 44% of grass N was transferred from clover. As a result, from 1.5 to 6.3 g N m^{-2} a^{-1} of clover N was transferred to grass. The NA method was found to be robust and can be applied to determine the N transfer in temporary grasslands established across a range of fertilisation treatments, including under moderate mineral N fertilisation. The NA procedure that agreed best with the average results from all NA and LAB procedures requires only the δ^{15}N of the shoots of clover and grass growing in the mixture.

1. Introduction

Nitrogen (N) transfer from legumes to non-legumes in grasslands is an often-overlooked input of symbiotically fixed N (Chalk et al., 2014; Høgh-Jensen, 2006). Nitrogen transfer has been defined as the N movement from a living plant to an associated plant (Thilakarathna et al., 2016). The proportion of non-legume N transferred from legumes varies widely in grasslands, from 0% to more than 70% of the receiver plant's N (Thilakarathna et al., 2016). In mown legume-grass mixtures where shoots are usually removed at harvest, the definition is often restricted to the transfer of N from legume belowground N (source) to the non-legume (receiver) (Peoples et al., 2015). The main process underlying this N transfer is the mineralisation of N from decomposing legume roots (Trannin et al., 2000) and rhizodeposition (Lesuffleur et al., 2013). Rhizodeposition is composed of root exudates, border cells, and debris (Uren, 2007).

Both ^{15}N natural abundance (NA) and legume ^{15}N labelling (LAB) methods have been used to determine N transfer from clover to associated non-legumes (Chalk et al., 2014). Both are based on a difference in ^{15}N isotopic signatures between the two N sources of the non-legume receiver plant. The two sources are (i) legume N and (ii) plant available soil N. The ^{15}N signature of the N taken up by the non-legume will fall between the contrasting ^{15}N signatures of these N sources (Daudin and Sierra, 2008; Ledgard et al., 1985). The NA method uses a naturally occurring small difference in ^{15}N between these sources (Daudin and Sierra, 2008; Shearer and Kohl, 1986), is based on a typical two-source mixing model (Wiederhold, 2015), and uses the delta (δ) notation to express a natural ^{15}N enrichment relative to the ^{15}N natural abundance of atmospheric N_2 (Robinson, 2001). The LAB method strongly increases

* Corresponding author.
E-mail address: astrid.oberson@usys.ethz.ch (A. Oberson).
[1] Current address: Landesbetrieb Landwirtschaft Hessen, Friedberg (Hesse), Germany.

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Abbreviations

AGNdfa	Amount of grass nitrogen derived from atmosphere via transfer of symbiotically fixed clover nitrogen
AGNtfc	Amount of grass nitrogen transferred from clover
BIOORG1	Bio-organic cropping system fertilised with animal manure at half level
BIOORG2	Bio-organic cropping system fertilised with animal manure at regular level
CONMIN2	Conventional cropping system fertilised with mineral fertiliser at regular level
DOK	Long-term field experiment comparing bio-**D**ynamic, bio-**O**rganic, and conventional (**K**) cropping systems
EAF	Excess atom fraction
LAB	^{15}N labelling method, with LAB I, LAB II, and LAB III denoting different labelling and calculation procedures
LAB1Ex1	Mixture-microplot ^{15}N labelled during year 1 and excavated at the end of year 1
LAB1Ex2	Mixture-microplot ^{15}N labelled during year 1 but excavated at the end of year 2
LAB2Ex2	Mixture-microplot ^{15}N labelled during year 2 and excavated at the end of year 2
NA	^{15}N natural abundance, with procedures NA I to NA IV differing in proxies representing the ^{15}N signature of the N sources of grass to calculate GNtfc
NAEx1	Microplot under ^{15}N natural abundance excavated at end of year 1; subscript m denotes microplot planted with clover-grass mixture, subscript p denotes pure grass
NAEx2	Microplot under ^{15}N natural abundance excavated at end of year 2; for subscripts see explanation NAEx1
NOFERT	Unfertilised control treatment
PCNdfa	Proportion of clover nitrogen derived from atmosphere via symbiotic N_2 fixation
PGNtrfc	Proportion of grass nitrogen transferred from clover

the ^{15}N signature of legume N by labelling the legume with a ^{15}N enriched tracer (Ledgard et al., 1985), resulting in a manyfold ^{15}N enrichment in legume N compared to the natural ^{15}N abundance of plant available soil N. With this method, the impact of the ^{15}N signature of unlabelled plant available soil N on the ^{15}N signature of the non-legume is usually negligible.

Nitrogen-15 shoot labelling is the most common enrichment technique to determine N transfer, usually via labelling the stems or leaves of the legumes (Chalk et al., 2014). Nitrogen-15 labelled plants are not uniformly enriched in ^{15}N, usually with higher ^{15}N enrichments in shoots than in roots (Gardner et al., 2012; Gasser et al., 2015; Giller et al., 1991; Hammelehle et al., 2018). Therefore, because roots are the main source of N transfer in mown grasslands (Thilakarathna et al., 2016), ^{15}N enrichment of the legume root is used in calculation protocols (Giller et al., 1991; Johansen and Jensen, 1996).

The NA method has increasingly been used over the past 20 years to determine N transfer (Peoples et al., 2015; Sierra et al., 2007). Advantages compared to ^{15}N labelling are elimination of costs for the ^{15}N enriched tracer, time savings for the laborious labelling, time and cost savings for unneeded delineating of the soil under ^{15}N labelled plants into subplots to prevent contamination of the main plot with ^{15}N enriched tracer, and the non-invasiveness. Further, the NA method provides continuous labelling via natural N uptake pathways of N from the soil solution or via symbiotic N_2 fixation (Shearer and Kohl, 1986), whilst the LAB method applies a pulse labelling to legume leaves or stems in the form of a ^{15}N labelled molecule, which leads to an immediate sharp increase in the ^{15}N signature (Putz et al, 2011). Concerns have been raised as to whether the isotopic fractionation occurring during legume N transformation in soil and N uptake by the receiver plant is considered correctly with the NA method (Høgh-Jensen, 2006; Peoples et al., 2015). Thus, the question remains as to which NA proxy to use to properly determine the $\delta^{15}N$ of legume N and plant available soil N sources.

The $\delta^{15}N$ of the source N transferred from the legume is usually represented by the $\delta^{15}N$ of the legume shoots (Moyer-Henry et al., 2006; Oberson et al., 2013; Schipanski and Drinkwater, 2012). However, legume shoots could be depleted in ^{15}N compared to roots due to isotopic fractionation within the legume (Högberg, 1997; Shearer and Kohl, 1986). Since belowground legume N is assumed to be the main source of N transfer to associated non-legumes in mown swards, the impact of using the $\delta^{15}N$ value of legume roots versus shoots should be examined (Peoples et al., 2015).

The $\delta^{15}N$ of plant available soil N is usually represented by the $\delta^{15}N$ of a non-legume that has not been affected by N transfer from legumes. Examples are the $\delta^{15}N$ of a pure stand reference plant (Høgh-Jensen and Schjoerring, 1994; Nygren and Leblanc, 2015) or the initial $\delta^{15}N$ of a grass shoot obtained at the first harvest of a newly established mixture (Oberson et al., 2013). The latter approach assumes that until the first harvest grass has not yet taken up N transferred from clover and that this $\delta^{15}N$ value represents that of plant available soil N during the subsequent growth of the mixture (Oberson et al., 2013). Plant available soil N comprises both fertiliser N and mineralised soil N (Oberson et al., 2013; Shearer and Kohl, 1986), and their combination in plant available soil N results in the $\delta^{15}N$ of the non-legume reference plant. While mineralised soil N is usually naturally enriched in ^{15}N (Handley and Raven, 1992; Shearer and Kohl, 1986), the $\delta^{15}N$ of fertiliser-derived N could be either naturally enriched in the case of animal manure or depleted in the case of mineral N fertiliser (Choi et al., 2017). Thus, dominance of mineral fertiliser N in the plant available soil N pool could lower its $\delta^{15}N$ to nearly that of the $\delta^{15}N$ of atmospheric N_2 (0‰). In a long-term field experiment under organic and conventional cropping for three decades, the $\delta^{15}N$ of total soil N, which is dominated by organic N, ranged from 6.3‰ to 7.4‰ due to different fertilisation regimes, with either none, mineral, and/or animal manure (DOK, bio-Dynamic, bio--Organic, and conventional (Konventionell)) (Oberson et al., 2007, 2013). Long-term mineral N fertilisation resulted in a relatively high $\delta^{15}N$ of 6.3‰ of total soil N (Oberson et al., 2007); the NA method should be applicable in this case so long as a significant proportion of plant available soil N derives from mineralisation. Oberson et al. (2013) applied the NA method to derive N transfer from clover to associated grass in field plots of the DOK experiment using the $\delta^{15}N$ value of the legume shoots and the $\delta^{15}N$ value of the initial grass shoots to determine plant available soil N at the first harvest of the newly established mixture. However, the robustness of the NA method representing the $\delta^{15}N$ values of the two sources has not yet been examined under the application of different proxies. In addition, NA has not been compared with a ^{15}N LAB method under the same environmental and management conditions.

Hence, our objectives were to i) evaluate the NA method in a model red clover-perennial ryegrass mixture under different fertilisation regimes (none, animal manure, synthetic mineral fertiliser) during two consecutive years using different proxies to represent the $\delta^{15}N$ values of the two sources, and comparing these results to those obtained with LAB applied in the same experiment; and to ii) determine the impact of these fertilisation treatments on the amount of grass N transferred from clover (AGNtfc), including the proportion of clover N therein derived from atmosphere via symbiotic N_2 fixation (PCNdfa).

Whilst we expected that our study may result in a refined NA procedure regarding the sources' $\delta^{15}N$ values, we hypothesised that i) the NA method is also applicable when mineral N fertilisers with a $\delta^{15}N$ of around 0‰ are applied, since the $\delta^{15}N$ of mineralised soil N will increase the $\delta^{15}N$ of the plant available soil N source; and that ii) different forms and amounts of N fertiliser will influence the proportion of grass N

transferred from clover (PGNtfc) and, thus, the AGNtfc.

2. Materials and Methods

2.1. DOK experiment and microplot study

We conducted a microplot study in field plots of the DOK long-term experiment (Mayer et al., 2015) from March 2011 to November 2012. The experiment is located in Therwil near Basel (CH) at 307 m above sea level (7°33′ E, 47°30′ N) on a haplic Luvisol developed from alluvial loess deposits (Fließbach et al., 2007). It has compared bio-**D**ynamic, bio-**O**rganic, and conventional cropping systems at two fertilisation levels since 1978 (Mayer et al., 2015). The experiment also includes an unfertilised control treatment (NOFERT) and a conventional mineral fertilised reference treatment (CONMIN2). We examined four treatments (in increasing order of N supply): NOFERT, bio-organic at half (BIOORG1), bio-organic at regular level of fertilisation (BIOORG2), and a conventional mineral fertilised treatment at regular level of fertilisation (CONMIN2). In BIOORG2, regular level of fertilisation represents the average value of Swiss organic farms corresponding to a manure nutrient amount of 1.4 livestock units per ha; and in CONMIN2 the level is in accordance with Swiss national fertilisation guidelines (Flisch et al., 2009). CONMIN2 receives exclusively synthetic water-soluble mineral fertilisers while bio-organic receives solid and liquid cattle manure (Table 1, Hammelehle et al., 2018). Fertilisers' $\delta^{15}N$ signatures were 6.7‰ for farmyard manure and 10‰ for slurry (Oberson et al., 2013) with both fertilisers applied to bio-organic, and 0.5‰ for calcium ammonium nitrate applied to CONMIN2 (Table 1). Cropping systems in clover-grass differ almost exclusively in fertilisation regime but not in crop protection. Our study focuses on the impact of fertilisation on the use of NA to determine N transfer; therefore, the term *fertilisation treatment* will be used instead of cropping system hereafter. The microplots, i.e. subplots being delineated from the main plot by PVC tubes, were installed in the regular clover-grass sward of the DOK experiment and were cultivated with a model red clover-perennial ryegrass mixture (mixture-microplots, details see Hammelehle et al., 2018).

Seven microplots were installed in each field plot, resulting in a design of 7 microplots per replicate x 4 replicates x 4 treatments = 112 microplots. Per replicate, five microplots were cultivated with the model red clover-perennial ryegrass mixture and two with pure grass (Fig. 1). Microplots were delineated with PVC tubes (height: 0.3 m, diameter: 0.375 m, resulting in an area of 0.11 m^2), which were driven into the soil to a depth of 0.25 m. Subsequent to propagation in the greenhouse, 11 red clover (*Trifolium pratense* L., cv. Dafila) and 20 (31 in pure grass) perennial ryegrass (*Lolium perenne* L., cv. Lacerta) seedlings were transplanted to each microplot on March 29, 2011 (300 plants m^{-2}). To determine N transfer by LAB, clover was ^{15}N multiple-pulse leaf labelled (see below) in three of five mixture-microplots per field plot (LAB microplots, Fig. 1b + c). Clover remained unlabelled in the additional two mixture-microplots; these were used to determine N transfer (Fig. 1a) and symbiotic N_2 fixation by NA. Grass always remained unlabelled and clover was only cultivated in mixture. To avoid ^{15}N contamination of NA microplots, NA and LAB microplots were located in two different sub-units per plot. Clover and grass were harvested separately at four times during cultivation year 1 (2011, in the following termed *year 1*) and at five times during cultivation year 2 (2012, in the following termed *year 2*) (Fig. 1). One out of 3 LAB microplots (LAB1Ex1) and 2 out of 4 NA microplots (NAEx1 with mixture [$NAEx1_m$] and with pure stand [$NAEx1_p$]) were excavated at the end of year 1 and the remaining microplots (LAB1Ex2, LAB2Ex2, $NAEx2_m$, and $NAEx2_p$) were excavated at the end of year 2 (Fig. 1). Under the climatic conditions of the experimental site, the growing season of clover- grass mixtures begins in early spring, with the first harvest in May and the last harvest in October.

2.2. Nitrogen-15 labelling

Generally, about two weeks before an upcoming harvest (see Fig. 1b + c), each clover plant per LAB microplot was leaf labelled with a tracer solution containing urea with an atom fraction ^{15}N (see Equation (6)) of 990 000 ppm (ReseaChem, CH). The ^{15}N label input was adapted to the expected clover N uptake by using the growth pattern of a previously

Table 1
Nutrient inputs to microplots during the present study [a] and to corresponding field plots of the DOK long-term experiment (numbers in parentheses) [b], ^{15}N natural abundance isotopic signatures ($\delta^{15}N$) of the nitrogen fertilisers, and the nutrient status of the soil of the experiment.

Treatment	Mineral N [c]		Total N		Phosphorus		Potassium		N fertiliser	Soil [d,e,f]	SON [d]	P (CO_2) [f]	K (CO_2) [f,g]
	Average annual nutrient input [g m^{-2}]								$\delta^{15}N$ (‰)		[g kg^{-1}]	[mg kg^{-1}]	
NOFERT	zero	–	(–)	–	(–)	–	(–)	–	(–)	–	9.1	1.37	0.3[c]	2.8[c]
BIOORG1 [h,i]	low	1.0	(1.5)	3.2	(4.5)	0.7	(1.2)	9.8	(8.4)	6.7 [j]/10 [k]	8.8	1.44	0.5[b]	8.0[b]
BIOORG2 [h,i]	low	2.0	(2.9)	6.5	(8.9)	1.4	(2.5)	19.5	(16.7)	6.7 [j]/10 [k]	9.1	1.58	0.8[ab]	15.6[a]
CONMIN2 [l,m]	medium	12.0	(12.2)	12.0	(12.2)	2.0	(3.8)	16.0	(24.8)	0.5	7.8	1.42	1.0[a]	13.1[a]
SEM		–	(–)	–	(–)	–	(–)	–	(–)	–	0.2	0.03	0.1	1.2
p		–	(–)	–	(–)	–	(–)	–	(–)	–	n.s.	n.s.	***	***

Fertilisation treatments: NOFERT: unfertilised control, BIOORG1 and BIOORG2: bio-organic cropping systems with animal manure at half and regular level, CONMIN2: conventional cropping system with mineral fertiliser at regular level; mean of $n = 4$ (CONMIN2: $n = 3$) and *SEM* of $n = 15$; *t*-test LSD, $\alpha < 0.05$; n.s. and *** represent p values > 0.05 (not significant) and < 0.001, respectively; same letters indicate no significant difference between treatments; SON: soil organic nitrogen; P: phosphorus; K: potassium.

[a] Average annual input of the applied nutrients to the model-mixture in microplots between August 2010 and 2012 [i, l)]

[b] Average annual input of the applied nutrients to the clover-grass mixture in field plots between 1978 and 2012.

[c] Ammonium-N and nitrate-N.

[d] Data from baseline sampling 10.3.2011 (FlashEA 1112 NC Analysers, Thermo Fisher Scientific Inc., US), soil layer 0–0.25 m.

[e] One-factorial mixed-effect model: treatment + error (clay content).

[f] Data were Box-Cox transformed prior to statistical analysis.

[g] Data from baseline sampling 10.3.2011, extraction with CO_2 saturated water (Flisch et al., 2009), soil layer 0–0.25 m.

[h] Microplots were fertilised with manure (6.6 kg N ha^{-1} applied before seeding clover-grass into field plots on August 23rd, 2010) and with slurry (year 1: 1.5 g N m^{-2} applied before harvest 2; year 2: 3.1 g N m^{-2} applied before harvest 1 and 1.7 g N m^{-2} applied before harvest 2).

[i] BIOORG1 and BIOORG2 in field plots received animal manure from 0.7 (0.6 until 1992) and 1.4 (1.2 until 1992) livestock units ha^{-1} a^{-1}, respectively.

[j] Manure.

[k] Slurry (data from Oberson et al., 2013).

[l] Nitrogen was applied to microplots as calcium ammonium nitrate (3 g N m^{-2} applied before seeding clover-grass into field plots on August 23rd, 2010; year 1: 4 g N m^{-2} applied before harvest 2, 3 g N m^{-2} applied before harvest 3 and 4; year 2: 4 g N m^{-2} applied before harvest 5, 3 g N m^{-2} applied before harvest 6, 7, and 8).

[m] CONMIN2 since 1985 (average from 1985 to 2012), unfertilised from 1978 to 1984.

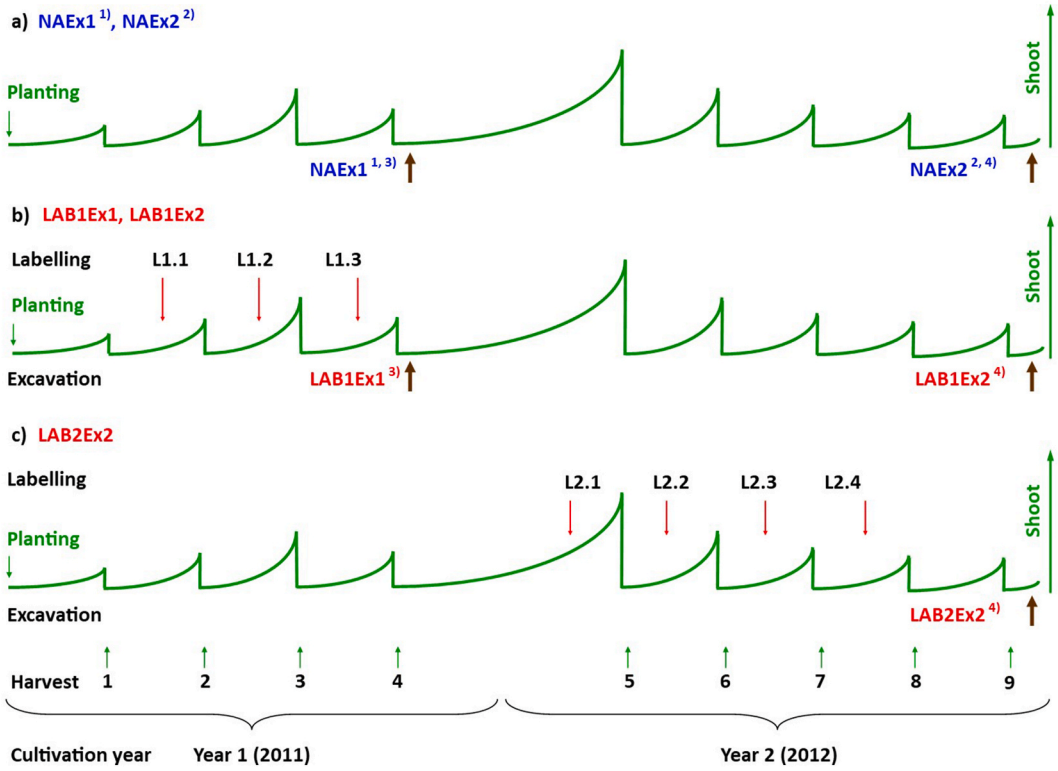

Fig. 1. Design of the microplot study: management timeline of a) ^{15}N natural abundance (NA) microplots (unlabelled) with the mixture and pure grass excavated at the end of year 1 (NAEx1) or year 2 (NAEx2), ^{15}N labelled (LAB) mixture-microplots b) labelled during year 1 and excavated at the end of year 1 (LAB1Ex1) or at the end of year 2 (LAB1Ex2), or c) labelled during year 2 and excavated at the end of year 2 (LAB2Ex2). The harvested shoot is represented qualitatively by the height of the green line.
[1)] One microplot with the mixture ($NAEx1_m$) and one with pure grass ($NAEx1_p$).
[2)] One microplot with the mixture ($NAEx2_m$) and one with pure grass ($NAEx2_p$).
[3)] Excavated between 24.10. and 28.11.2011.
[4)] Excavated between 24.10. and 1.11.2012.

cultivated clover-grass mixture of the DOK experiment (Oberson et al., 2013). One single trifoliate clover leaf per plant was squashed manually while remaining on the plant. The squashed leaf was inserted into a 2 ml vial, tracer solution was pipetted into the vial, and the vial with the inserted leaf was sealed airtight using Terostat IX (Henkel, DE) (for details see Hammelehle et al., 2018). Vials and inserted leaves with petioles were removed from the clover plants within 72 h after starting the labelling event. Tracer solution was generally absorbed within 24 h. To prevent N transfer from decomposing clover shoot material, litter was collected weekly from the soil. Microplots LAB1Ex1 and LAB1Ex2 were labelled before harvests 2, 3, and 4 during year 1 while microplots of LAB2Ex2 were labelled before harvests 5, 6, 7, and 8 during year 2 (Fig. 1b + c).

2.3. Sampling and processing of plant samples

The harvested shoots (harvested biomass) were cut 0.05 m above the ground at each harvest (see Fig. 1) using manual garden shears and separated into grass and clover. At the ends of year 1 (NAEx1 and LAB1Ex1) and year 2 (NAEx2, LAB1Ex2, and LAB2Ex2), the stubble and the roots (standing biomass) were also quantified. Stubble was collected separately for clover and grass by cutting the remaining aboveground biomass at the soil surface level after the last harvest and before excavating the soil contained in the microplots. Subsequently, roots were separated from the soil and divided into clover and grass roots by manual collections (macro roots), sieving with a 3 mm sieve (root fragments), and extraction by subsequent sieving with a 0.5 mm sieve (rootlets) (for details see Hammelehle et al., 2018). Shoots, stubble, and roots were dried at 60 °C for 72 h before dry matter determination. Dried plant parts were then ground to a fine powder using a centrifuge mill (Retsch GmbH, DE). Nitrogen concentration and isotope ratio $^{15}N/^{14}N$ were analysed using a FlashEA 1112 NC analyser coupled with a ConFlo IV universal continuous flow interface to a DELTA V isotope ratio mass spectrometer (Thermo Fisher Scientific Inc., US). Isotope ratios are reported in the conventional notation with respect to atmospheric N_2 (AIR) standard. The mass spectrometer was calibrated with the International Atomic Energy Agency reference materials IAEA-N-1 ($\delta^{15}N = +0.45‰$), IAEA-N-2 ($\delta^{15}N = +20.41‰$) and IAEA-NO-3 ($\delta^{15}N = +4.72‰$). Reproducibility of the measurements was better than 0.2‰ (Bernasconi, 2014, oral communication).

2.4. Calculation of grass N transferred from clover

2.4.1. Nitrogen-15 natural abundance method

The NA method to determine the PGNtfc relies on a two-source mixing model (Equation (1)), with source 1 *clover N* and source 2 *plant available soil N* and the receiver *grass N* (adapted from Daudin and Sierra, 2008; Høgh-Jensen and Schjoerring, 1994):

$$\mathrm{PGNtfc} = \frac{\delta^{15}\mathrm{N\ plant\ available\ soil\ N} - \delta^{15}\mathrm{N\ grass\ N}}{\delta^{15}\mathrm{N\ plant\ available\ soil\ N} - \delta^{15}\mathrm{N\ clover\ N}} \quad (1)$$

The NA ^{15}N isotopic signature in the notation of delta values ($\delta^{15}N$, ‰) is calculated as the difference in relative isotopic ratio of a sample compared to a standard (Robinson, 2001), i.e., atmospheric N_2:

$$\delta^{15}N = \left(\frac{\frac{^{15}N}{^{14}N}\text{sample} - \frac{^{15}N}{^{14}N}N_2}{\frac{^{15}N}{^{14}N}N_2} \right) x1000 \quad (2)$$

with the isotopic ratio $^{15}N/^{14}N$ of N_2 as 3.6765×10^{-3} (Högberg, 1997).

We compared combinations of different proxies representing the $\delta^{15}N$ of the sources *clover N* and *plant available soil N* and of the receiver *grass in mixture*, resulting in four NA procedures (Table 2). For clover N, we used the $\delta^{15}N$ of either the clover shoots from the respective harvest (Moyer-Henry et al., 2006; Oberson et al., 2013; Schipanski and Drinkwater, 2012) or of the clover roots sampled at the end of the year of interest (Nygren and Leblanc, 2015). For plant available soil N, we used the $\delta^{15}N$ determined either from the pure grass harvested shoots (Høgh-Jensen and Schjoerring, 1994), the entire pure grass (weighed $\delta^{15}N$ of the harvested shoots comprising all harvests of the year of interest and the stubble and roots sampled at end of the same year) (Nygren and Leblanc, 2015), or the shoots of harvest 1 of the grass grown in the mixture (Oberson et al., 2013). For the receiver grass in mixture, we used either the $\delta^{15}N$ of the shoots of the respective harvest (Høgh-Jensen and Schjoerring, 1994; Oberson et al., 2013) or of the entire plant (weighed $\delta^{15}N$ of the harvested shoots comprising all harvests of the year of interest and of the stubble and roots sampled at end of the same year) (Moyer-Henry et al., 2006). The PGNtfc was calculated for year 1 and for year 2. Exemplified for the calculations of the PGNtfc for the different NA procedures, the specific equation for the procedure NA III (see Table 2) was:

$$\text{PGNtfc (t)} = \frac{\sum_{i}^{n} \frac{\delta^{15}N\ \text{grass}_m SH_1 - \delta^{15}N\text{grass}_m SH_n}{\delta^{15}N\text{grass}_m SH_1 - \delta^{15}N\text{clover}_m SH_n} \times N\ \text{grass}_m SH_n\ [g\ m^{-2}]}{\sum_{i}^{n} N\ \text{grass}_m SH_n\ [g\ m^{-2}]} \quad (3)$$

Equations S1 to S3.

2.4.2. Nitrogen-15 labelling method

The LAB method to determine the PGNtfc also relies on a two-source mixing-model. However, while source 2 *plant available soil N* remained at ^{15}N natural abundance, source 1 *clover N* became ^{15}N enriched by labelling. The PGNtfc was calculated by relating the ^{15}N enrichment of grass in mixture (receiver) to that of the root of the associated clover (source) (Giller et al., 1991):

$$\text{PGNtfc} = \frac{\text{EAF } ^{15}N \text{ grass}}{\text{EAF } ^{15}N \text{ clover root}} \quad (4)$$

with EAF ^{15}N calculated by subtracting the atom fraction ^{15}N of an unlabelled sample (i.e., at ^{15}N natural abundance) from the atom fraction ^{15}N of the labelled sample (Coplen, 2011):

$$\text{EAF } ^{15}N = \text{atom fraction } ^{15}N_{\text{labelled sample}} - \text{atom fraction } ^{15}N_{\text{NA sample}} \quad (5)$$

Atom fraction ^{15}N is calculated (Coplen, 2011) according to

$$^{15}N \text{ atom fraction (ppm)} = \frac{^{15}N}{^{14}N + ^{15}N} \times 1\,000\,000 \quad (6)$$

The use of ^{15}N enrichment of the root in Equation (4) assumed that the roots were homogeneously enriched in ^{15}N over time and space (adapted from Jensen, 1996; Sawatsky and Soper, 1991). The PGNtfc was determined based on Equation (4) using receiver grass parts of the year of interest. The ^{15}N enrichment of grass was calculated as the weighted mean of the ^{15}N enrichment of the shoots of harvest *i* to *n* of year *t* and the stubble and the roots sampled at the end of year *t*:

$$\text{EAF } ^{15}N \text{ grass (t)} = \frac{\sum_{i}^{n} \text{EAF } ^{15}N \text{ shoot of harvest}_i \text{ x N shoot of harvest}_i\ [g\ m^{-2}] + \text{EAF } ^{15}N \text{ stubble(t) x N stubble(t)}[g\ m^{-2}] + \text{EAF } ^{15}N \text{ root(t) x N root(t)}[g\ m^{-2}]}{\sum_{i}^{n} N \text{ shoot of harvest}_i\ [g\ m^{-2}] + N \text{ stubble(t)}\ [g\ m^{-2}] + \text{root(t)}\ [g\ m^{-2}]} \quad (7)$$

with $\text{grass}_m\ SH_1$ denoting the grass in mixture shoots of harvest 1, $\text{clover}_m\ SH_n$ denoting the clover in mixture shoots of harvest n with $i = 2$ and $n = 4$ for $t =$ year 1 and $i = 5$ and $n = 9$ for $t =$ year 2, and t denoting the year of cultivation. Further equations with the specific proxies inserted into Equation (1) are shown in supplemental material,

For the calculation of PGNtc with LAB, procedures differed in labelling strategies and/or calculation (Table 3). For year 1, PGNtfc was obtained using the same labelling procedure applied during year 1 to two different sets of microplots (LAB1Ex1, LAB1Ex2), and using the ^{15}N enrichment of the clover roots as follows:

Table 2
Plants and plant parts used with the ^{15}N natural abundance method representing the $\delta^{15}N$ isotopic signature of the two sources *clover N* and *plant available soil N,* and the N receiver *grass in mixture.*

Procedure	$\delta^{15}N$ of the source/receiver was determined [a] from the			
	Source 1	Source 2	Receiver	
	Clover N	Plant available soil N	Grass in mixture	Calculation
NA I	Harvested shoot [b]	Harvested shoot of grass_p	Harvested shoot [b]	Equation S1
NA II	Root [c]	Entire pure grass [d]	Entire plant [d]	Equation S2
NA III	Harvested shoot [b,e]	Shoot of harvest 1 of grass_m	Harvested shoot [b,e]	Equation 3
NA IV	Root [c]	Shoot of harvest 1 of grass_m	Harvested shoot [b,e]	Equation S3

Grass_p: pure grass; grass_m: grass in mixture.
[a] Based on Equation 1.
[b] Comprising respective harvests (see Fig. 1).
[c] Sampled at the end of the year of interest (see Fig. 1).
[d] Comprising the harvested shoot, the stubble[c)], and the root[c)]
[e] Shoot of harvest 1 was not considered.

Table 3
Microplots (MP) used with the ^{15}N labelling method for determination of the excess atom fraction (EAF) ^{15}N of the source 1[a] *clover N* (using clover roots) and of the N receiver grass in mixture.

Procedure	EAF ^{15}N was determined from the				Calculation	
	clover root (source 1) of MP		grass in mixture (receiver) of MP		Yr1	Yr2
	Yr1	Yr2	Yr1	Yr2		
LAB I	LAB1Ex1	LAB2Ex2	LAB1Ex1	LAB2Ex2	Equation 8	Equation 10
LAB II	LAB1Ex1	LAB1Ex2	LAB1Ex2	LAB1Ex2	Equation 9	Equation 11
LAB III	–	LAB1Ex1, LAB1Ex2 [b]	–	LAB1Ex2	–	Equation 12

[a] Source 2 *plant available soil N* remained unlabelled.
[b] Geometric mean of the roots' EAF ^{15}N from microplots LAB1Ex1 and LAB1Ex2.

i) Procedure LAB I, using shoots, stubble and roots from LAB1Ex1 with roots and stubble sampled at the end of year 1 (Fig. 1):

$$\text{PGNtfc (year 1)} = \frac{\text{EAF } ^{15}\text{N grass (LAB1Ex1)}}{\text{EAF } ^{15}\text{N clover root (LAB1Ex1)}} \quad (8)$$

with the EAF ^{15}N of grass calculated according to Equation (7), with $t = 1$, $i = 2$ (harvest 1 remained unlabelled, see Fig. 1), and $n = 4$.

ii) Procedure LAB II, using shoots from LAB1Ex2, but the stubble and roots from LAB1Ex1:

$$\text{PGNtfc (year 1)} = \frac{\text{EAF } ^{15}\text{N grass (LAB1Ex2)}}{\text{EAF } ^{15}\text{N clover root (LAB1Ex1)}} \quad (9)$$

with the EAF ^{15}N of grass calculated according to LAB I.

Three procedures were applied for the PGNtfc of year 2 (Table 3):

i) LAB I similar to year 1 (Equation (8)) with labelling in microplots of LAB2Ex2 during year 2:

$$\text{PGNtfc (year 2)} = \frac{\text{EAF } ^{15}\text{N grass (LAB2Ex2)}}{\text{EAF } ^{15}\text{N clover root (LAB2Ex2)}} \quad (10)$$

with the EAF ^{15}N of grass calculated according to Equation (7), with $t = 2$, $i = 6$ (harvest 5 remained unlabelled, see Fig. 1), and $n = 9$.

ii) LAB II similar to LAB II in year 1 (Equation (9)) using grass biomass from LAB1Ex2 sampled during year 2, but using the clover roots and the stubble sampled at the end of year 2 from LAB1Ex2:

$$\text{PGNtfc (year 2)} = \frac{\text{EAF } ^{15}\text{N grass (LAB1Ex2)}}{\text{EAF } ^{15}\text{N clover root (LAB1Ex2)}} \quad (11)$$

with the EAF ^{15}N of grass being calculated according to Equation (7), with $t = 2$, $i = 5$ (labelling was carried out in year 1, thus, harvest 5 had already been labelled, see Fig. 1), and $n = 9$.

iii) LAB III similar to LAB II (Equation (11)), except that EAF ^{15}N of the clover roots was calculated as the geometric mean of the clover roots' EAF ^{15}N sampled at the end of year 1 from LAB1Ex1 and sampled at the end of year 2 from LAB1Ex2:

$$\text{PGNtfc (year 2)} = \frac{\text{EAF } ^{15}\text{N grass (LAB1Ex2)}}{\text{EAF } ^{15}\text{N clover root (geometric mean of LAB1Ex1 and LAB1Ex2)}} \quad (12)$$

2.4.3. Amount of grass N transferred from clover

The amount of GNtfc (AGNfdc) for year t was obtained by multiplying the N uptake of grass in mixture with the PGNtfc:

$$\text{AGNtfc (t)} \left[\text{g m}^{-2}\right] = \text{PGNtfc (t)} \times \text{N uptake grass}_{\text{m}} \quad (13)$$

with N uptake calculated by multiplying the dry matter biomass production (harvested biomass plus standing biomass [stubble and root]) by the respective N concentration.

Equation (13) was applied for calculation of the AGNtfc using the PGNtfc obtained from any of the NA or LAB procedures (Table 2, Table 3), using the N uptake of the corresponding year and microplot. Only AGNtfc obtained with PGNtfc from NA III will be shown, as explained below. With NA III, the grass of harvest 1 grown in mixture was not considered in the N uptake because it was used to represent the source signature of plant available soil N (Table 2).

2.5. Determination of red clover N derived from the atmosphere

The PCNdfa was determined using the widely used NA method (Chalk et al., 2016; Shearer and Kohl 1986). In brief, it is also based on a two-source mixing model with $\delta^{15}N$ of plant available soil N and of atmospheric N as the two sources and $\delta^{15}N$ of clover as receiver:

$$\text{PCNdfa} = \frac{\delta^{15}\text{N}_{\text{plant available soil N}} - \delta^{15}\text{N}_{\text{clover}}}{\delta^{15}\text{N}_{\text{plant available soil N}} - \delta^{15}\text{N}_{\text{atmosphere}}} \quad (14)$$

with $\delta^{15}N$ plant available soil N represented by the $\delta^{15}N$ of a non-legume reference plant and $\delta^{15}N$ atmosphere represented by the harvested shoots of clover as completely reliant on symbiotic N_2 fixation, i.e., accounting for fractionation from fixation and transportation of atmospheric N (Shearer and Kohl, 1986; Unkovich et al., 1994). $\delta^{15}N$ of a non-legume reference plant was represented by harvest 1 of mixed grass, in agreement with the use of that proxy for $\delta^{15}N$ of available soil N in the transfer determination. The PCNdfa was determined separately for each harvest. We set the lowest ^{15}N signature of a harvested clover shoot as the $\delta^{15}N_{\text{atmosphere}}$ value (Eriksen and Høgh-Jensen, 1998; Oberson et al., 2013; Råberg et al., 2018; Unkovich et al., 2008). This harvested shoot was selected from 60 single harvested shoots (4 harvests x 15 microplots) for year 1 (−1.3‰) and from 75 single harvested shoots (5 harvests x 15 microplots) for year 2 (−1‰, which was in accordance with Oberson et al., 2013).

The PCNdfa for year t was determined as the weighted mean of the

Table 4
Plant dry matter biomass production (DMBP) of ^{15}N natural abundance mixture-microplots comprising the harvested shoots (harvested biomass) and the stubble and roots (standing biomass) of red clover and perennial ryegrass and the clover proportion of the mixtures' dry matter of the harvested shoots for year 1 and year 2. The harvested shoots of year 1 comprise the sum of harvests 1 to 4 and the harvested shoots of year 2 comprise the sum of harvests 5 to 9. Stubble and roots were sampled at the end of the year of interest.

Species	Treatment	Plant DMBP [g m^{-2}]			Harvested shoot [g m^{-2}]			Stubble [g m^{-2}]			Root [g m^{-2}]			Clover prop.		
		Year 1	Year 2	T	Year 1	Year 2	T	Year 1	Year 2	T	Year 1	Year 2	T	Year 1	Year 2	T
Clover	NOFERT	912	1218	1063 c	561	889	706 c	86 cd	52 d	69	260	260	260 b	88%	82%	85% b
	BIOORG1	1989	2450	2209 ab	1422	1882	1636 ab	163 b	104 bcd	133	369	392	380 a	93%	84%	90% a
	BIOORG2	2530	2852	2683 a	1720	2276	1978 a	244 a	80 cd	162	539	428	483 a	94%	85%	90% a
	CONMIN2	1552	2440	1971 b	1033	1901	1408 b	141 bc	95 cd	118	392	473	428 a	77%	77%	77% c
	Year	1689 B	2190 A		1097 B	1636 A		158 A	83 B		376	380		89% A	82% B	
	SEM/mean			150/2023[a]			122/1495 [b]			12.5/121			32/407 [b]			1% / 85% [c]
	Treatment (T)			***			***			**			**			***
	Year (Y)			*			***			***			n.s			***
	T x Y			n.s.			n.s.			*			n.s			n.s
Grass	NOFERT	257	395	321 c	75 e	200 c	123	32	67	47	149	108	128			
	BIOORG1	358	528	437 b	99 de	358 b	189	29	72	46	225	100	156			
	BIOORG2	407	639	508 b	119 d	407 ab	219	52	75	62	234	149	188			
	CONMIN2	713	773	742 a	308 b	572 a	420	57	84	69	342	125	222			
	Year	403 B	567 A		129	358		41 B	74 A		231 A	119 B				
	SEM/mean			36/501 [b]			32/259 [b]			6/62			16/179 [a]			
	Treatment (T)			***			***			n.s.			n.s			
	Year (Y)			***			***			***			***			
	T x Y			n.s.			*			n.s.			n.s.			

Year 1 from NAEx1; year 2 from NAEx2; two factorial mixed effect model (treatment x year + error [replication, row]); treatment's mean of $n = 4$ (CONMIN2: $n = 3$); n.s., *, **, and *** represents p values > 0.05 (not significant), <0.05, <0.01, and <0.001, respectively; t-test LSD, $\alpha < 0.05$; same letters indicate no significant difference between factors with lowercase letters referring to treatments and capital letters referring to years; for details of fertilisation treatments and statistics see Table 1.

[a] Data were square root transformed.

[b] Data were log transformed.

[c] Data were centred log-ratio transformed.

PCNdfa of harvest i to n according to:

$$\text{PCNdfa}\,(t) = \frac{\sum_{i}^{n} \text{PCNdfa shoot of harvest}_i \text{ x N clover shoot of harvest}_i\ [\text{g m}^{-2}]}{\sum_{i=1}^{n} \text{N clover shoot of harvest}_i\ [\text{g m}^{-2}]} \quad (15)$$

with $i = 1$ and $n = 4$ for t = year 1 and $i = 5$ and $n = 9$ for t = year 2.

This proportion was used to calculate the amount of grass N derived from the atmosphere via transfer of symbiotically fixed clover N (AGNdfa) by multiplying it with the AGNtfc.

In the results section, all proportions (PGNtfc, PCNdfa) are given in percentages, i.e., fractions calculated as shown above, multiplied by 100.

2.6. Experimental design and statistical analyses

The experimental design consisted of a split-split plot, which was embedded in the Latin square of the DOK experiment (Oberson et al., 2013, experimental design of the DOK experiment depicted in Fließbach et al., 2007). Data were fitted to a two-factorial mixed effect model (method and respective procedure x treatment + error [replication, row]), if not footnoted differently. The model was assumed to be significant above an α level of 0.05. The random factors *replication* and *row* are owed to the experimental design of the DOK experiment, where NOFERT and CONMIN2 on the one hand and BIOORG1 and BIOORG2 on the other hand are situated opposite one another (see Fig. 1 in Fließbach et al., 2007).

Normal distribution of Studentized residuals was tested with the Shapiro-Wilk test prior to the statistical analysis. If normal distribution was violated, data were transformed. Compositional data (e.g. proportions) were always centred log-ratio transformed (van den Boogaart and Tolosana-Delgado, 2013). Centred log-ratio transformation was carried out using CoDaPack version 2.01.15 (Thió-Henestrosa et al., 2009). Differences between least significant means of the factors ([method + procedure], treatment, etc.) were tested using a Student's *t*-test. Microplots located in the fourth replicate of CONMIN2 were excluded since deer had eliminated most of the clover in the mixture by harvest 2. Statistical analyses were carried out with the software R version 4.1.0 (R core team, 2021) and the R packages *lme4* version 1.1–29 (Bates et al., 2015) and *lmerTest* version 3.1–3 (Kuznetsova et al., 2017).

3. Results

3.1. Biomass production and N uptake by red clover and perennial ryegrass

We present the dry matter biomass production and N uptake data from the NA microplots (NAEx1 and NAEx2) since these data were the basis for NA III, which was the procedure selected for calculating AGNtfc and AGNdfa (Table 6). Reasons for this choice are given in the chapter "Little impact of different proxies for δ^{15}N of sources in ^{15}N natural abundance method". Dry matter biomass production and N uptake did not significantly differ between NA and LAB mixture-microplots of the same year (data not shown).

Under all treatments, biomass production of red clover was greater than that of perennial ryegrass (Table 4). The annual biomass production (harvested biomass plus standing biomass) averaged over both years was about 2000 g m^{-2} a^{-1} for clover and about 500 g m^{-2} a^{-1} for grass. Clover biomass production and harvested shoots were highest in organic treatments and lowest in NOFERT. Grass biomass production and harvested shoots were highest in CONMIN2 and also lowest in NOFERT.

The clover proportion in our model mixture was high (Table 4). The average clover proportion considering the harvested shoots of the mixture of both years was 85%. The two year average clover proportion differed significantly between the treatments, increasing in the order CONMIN2 (77%) < NOFERT (85%) < BIOORG1 = BIOORG2 (90%). Except in CONMIN2, the clover proportion decreased from year 1 to year 2.

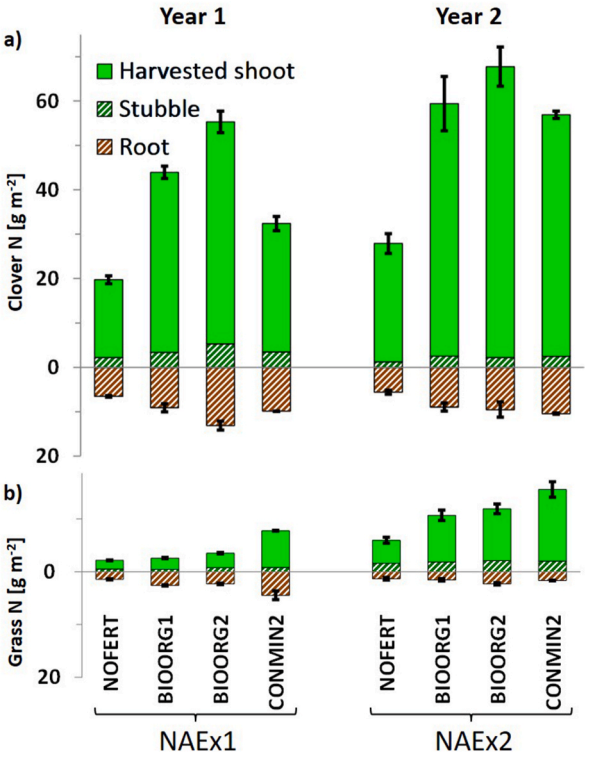

Fig. 2. Nitrogen uptake by a) red clover and b) perennial ryegrass cultivated in ^{15}N natural abundance mixture-microplots during year 1 and year 2.
The harvested shoots (harvested biomass) of year 1 comprise the sum of harvests 1 to 4 from NAEx1; the harvested shoots of year 2 comprise the sum of harvests 5 to 9 from NAEx2; the stubble and the roots (standing biomass) were sampled at the end of the year of interest; data are given in detail in Table S1; error bars represent ± one *SEM*; for details of fertilisation treatments and statistics see Table 1.

The N uptake of clover and grass in mixtures of the entire plant as well as the harvested shoots (Fig. 2) followed their patterns of dry matter biomass production (Table 4). Clover harvested shoot and root N uptake increased with fertilisation except in CONMIN2 (Fig. 2). Clover N uptake was higher during year 2 than year 1, as was the case for dry matter biomass production.

3.2. Nitrogen-15 isotopic signatures of plants

3.2.1. Natural abundance

Under ^{15}N natural abundance, δ^{15}N values of clover harvested shoots were always significantly lower than those of associated grass in mixture and in pure grass (Fig. 3). This also applied to the stubble and the roots, except in NOFERT and CONMIN2 at the end of year 1 (Table S2). Already at harvest 1, δ^{15}N of clover harvested shoots had dropped below the value of atmospheric N_2 (0‰), down to around −0.4‰. At ≤ −0.6‰, the lowest δ^{15}N was generally found between harvest 4 and harvest 6. Clovers' δ^{15}N signatures of the stubble (−0.1‰ to −1.0‰) and the roots (0‰ to −0.9‰) were within the range of those of the harvested shoots (0.3‰ to −0.9‰) (Fig. 3, Table S2 and Table S3).

The δ^{15}N of plant available soil N was represented either by that of the shoots of the corresponding harvest of pure grass or by that of the shoots of harvest 1 of grass in mixture (Table 2). The δ^{15}N of the shoots

of harvest 1 of grass in mixture did not significantly differ from the weighted mean of the pure grass proxies of treatments BIOORG1 and BIOORG2 in year 1 or of any treatment in year 2 (Table S4). In year 1, it was up to 2‰ higher in NOFERT, but 0.5‰ lower in CONMIN2 (Table S4).

The $\delta^{15}N$ of plant available soil N represented by the $\delta^{15}N$ of the shoots of grass in mixture shoot of harvest 1 was significantly higher than the $\delta^{15}N$ of clovers' harvested shoots and clovers' stubble and root sampled at the end of year 1 and year 2. Differences stayed relatively stable fluctuating 1.5‰–2.4‰ for CONMIN2, 4.3‰–5.2‰ for BIOORG2, 4.7‰–5.3‰ for NOFERT, and 4.6‰–5.8‰ for BIOORG1 (Fig. 3, Table S3).

The $\delta^{15}N$ of the harvested shoots of grass in mixture (receiver) generally decreased over time in year 1 (Fig. 3). The $\delta^{15}N$ of harvested shoots was significantly lower in CONMIN2 compared to the other treatments ($p < 0.001$). The $\delta^{15}N$ of total soil N under the mixture and under pure grass was significantly higher than that of the plant parts, from 2.9‰ to 5.7‰ higher compared to grass in mixture, 3.3‰–4.6‰ compared to pure grass, and 8.3‰–9.5‰ compared to clover in mixture (Fig. 3, Table S3). The difference in $\delta^{15}N$ under CONMIN2 was lower between the soil and clover, but higher between the soil and grass in mixture as well as in pure grass compared to the other treatments.

3.2.2. Labelling

Different labelling strategies and calculation procedures were used (as described in the Materials and Methods), which required different microplots. During year 1, labelling strategies were identical in LAB1Ex1 and LAB1Ex2 and resulted in similar EAF ^{15}N values of harvested shoots of clover and grass (Figs. 4 and 5). During year two, however, labelling strategies differed fundamentally, and in turn, so did the resulting EAF ^{15}N.

In LAB I, the PGNtfc was determined based on two different sets of microplots for year 1 and year 2, with clover plants labelled during the respective year (Table 3, Fig. 4). Clover was always more highly enriched in ^{15}N than the associated grass (Fig. 4). The EAF ^{15}N of clover plant parts quickly responded to the labelling, as expected. Clover harvested shoots' EAF ^{15}N fluctuated tremendously during year 1 (e.g., for CONMIN2 from 4583 ppm to 15 678 ppm, Fig. 4, Table S4) and during year 2 (e.g., for CONMIN2 from 2108 ppm to 13 504 ppm). The EAF ^{15}N of grass' harvested shoots increased steadily through the last labelling (Fig. 4). At the end of year 1, clover stubble and roots were enriched in ^{15}N comparable to the shoots of harvest 3 (Fig. 4, Table S5). At the end of year 2, clover stubble and roots were also comparably enriched in ^{15}N, but higher than shoots of harvest 9 and generally lower than shoots of harvests 6 and 8 (Fig. 4, Table S5).

To determine the PGNtfc using LAB II (Table 3), the EAF ^{15}N of the clover roots and all grass plant parts cultivated in microplot LAB1Ex2 were used. Because no labelling of clover was carried out in year 2 (Fig. 5), the EAF ^{15}N of clover was distinctly lower during year 2 than during year 1 (Fig. 5, Table S5). The main decline in EAF between years 1 and 2 occurred over winter (between harvests 4 and 5) followed by a slower decline between harvests 5 and 7. At the end of year 2, the EAF ^{15}N of the clover roots was lower than that of all clover plant parts of year 1 but higher than that of the clover shoots and stubble of year 2 (Fig. 5, Table S5).

The development of EAF ^{15}N of the grass that grew in association with the labelled clover in LAB1Ex2 changed over time with a peak between harvests 3 and 5 depending on the treatment (Fig. 5). Grass plant parts, except for the roots, had a lower EAF ^{15}N than corresponding clover plant parts during year 1 but higher than clover during year 2 ($p < 0.001$).

3.3. Proportions of N transfer

As averaged over all treatments, the PGNtfc determined by the different NA and LAB procedures ranged from 27% to 51% in year 1 (Table 5). Thereby, the average PGNtfc obtained by LAB procedures (51%) were not significantly different from those obtained with NA procedures (45%–48%), except for the lower PGNtfc obtained with NA I, where the $\delta^{15}N$ of the pure grass shoots was used to represent the $\delta^{15}N$ of plant available soil N (27%, Table 5). Also in year 2, NA and LAB methods resulted in similar PGNtfc, but they varied more between different LAB procedures (24%–54%) than between different NA procedures (37%–46%). LAB delivered both the significantly lowest and highest PGNtfc in year 2.

Averaged across all the NA and LAB procedures, fertilisation treatments did not significantly affect the PGNtfc in year 1, whilst in year 2, the PGNtfc of CONMIN2 was, at 34%, significantly lower than that of the organic treatments at around 46% (Table 5). In all methods and treatments, the PGNtfc was at about 44%, which was similar for years 1 and 2. The fast and easy in the field applicable procedure NA III, which does not need pure grass as reference and only requires the sampling of plant shoots (Table 2), resulted in similar PGNtfc means for all NA and LAB procedures, thus fitting best with the average (Table 5, Fig. S1). This was valid for both years and for all treatments, including CONMIN2. Hence, we used NA III for the calculation of the AGNtfc and the AGNdfa (Table 6).

3.4. Amount of grass N transferred from clover

The yearly AGNtfc ranged from 1.5 to 6.3 g m^{-2} (Table 6). It was affected by treatment and by year, with a signification interaction between treatment and year. While in year 1 up to three times more N was transferred from clover to grass in CONMIN2 (4.9 g m^{-2}) than in other treatments (1.5–2.1 g m^{-2}, Table 6), all treatments transferred similar AGNtfc in year 2. The AGNtfc of year 2 was significantly higher than that of year 1 except in the case of CONMIN2.

Clover obtained about 90% of its N via symbiotic N_2 fixation except in CONMIN2, which had significantly lower proportions of 69% in year 1 and 78% in year 2 (Table 6). Thus, most transferred N had originally been derived from the atmosphere. The resulting AGNdfa ranged from 1.4 to 3.3 g m^{-2} in year 1 and from 2.8 to 5.7 g m^{-2} in year 2 (Table 6). The AGNdfa was significantly lower in NOFERT than in other treatments.

4. Discussion

4.1. Little impact from proxies with ^{15}N natural abundance method

The NA method has significant advantages over LAB in terms of labour, costs, and disturbances to the plants. However, it depends on the correct representation of the $\delta^{15}N$ signature of both N sources of the grass, which include both N transferred from clover and plant available soil N (Peoples et al., 2015).

The $\delta^{15}N$ of the clover shoots is usually used to represent the ^{15}N signature of N transferred from clover. Since the shoots were found to be depleted in ^{15}N compared to the roots (Huss-Danell et al., 2007; Peoples et al., 2015), and because clover roots are the main source of N transferred from clover (Peoples et al., 2015; Thilakarathna et al., 2016), we compared the use of the $\delta^{15}N$ of the clover shoots from the respective harvest with that of the roots to determine the PGNtfc. We found few differences between the $\delta^{15}N$ of the roots and the harvested shoots (Fig. 3). In turn, the effect of different proxies of N transferred from clover on the estimates of PGNtfc were non-significant except for year 1 when we used clover harvested shoots in combination with pure grass harvested shoots as a proxy for plant available soil N (NA I, Tables 2 and 5).

Plant available soil N comprised fertiliser N and mineralised soil N (Shearer and Kohl, 1986), as fertilisers were applied during our experimental period (Table 1). The contribution of each may have varied over time and space; temporally between the harvests and spatially between the separately located microplots used for the mixture and the pure grass (Fig. 1). Therefore, we compared the use of the $\delta^{15}N$ of the respective

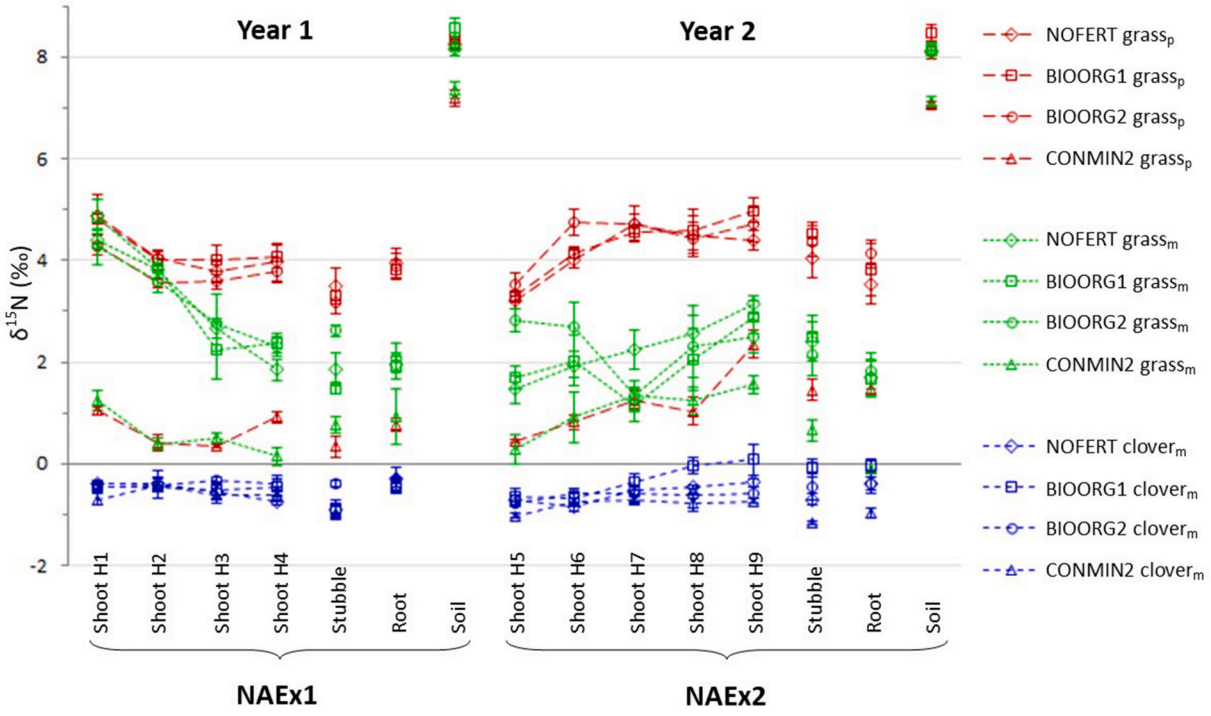

Fig. 3. Temporal development of the ^{15}N natural abundance signatures of clover in mixture ($clover_m$, proxy for source 1), pure grass ($grass_p$, proxy for source 2, i.e., plant available soil N), grass in mixture ($grass_m$, receiver) plant parts, and the soil (0–0.25 m) of the mixture, and of pure grass from NA microplots. Note that the shoots of harvest 1 (H1) of the grass in mixture were used as proxy for the $\delta^{15}N$ of source 2 in procedures NA III and NA IV (here we show only the H1 from for year 1 of NAEx1).
Year 1 data from microplot NAEx1 with roots and soils excavated at the end of year 1; data are given in detail in Table S2; year 2 from microplot NAEx2 with roots and soils excavated at the end of year 2, data including for year 1 are given in detail in Table S3; error bars represent ± one *SEM*; for details of fertilisation treatments see Table 1.

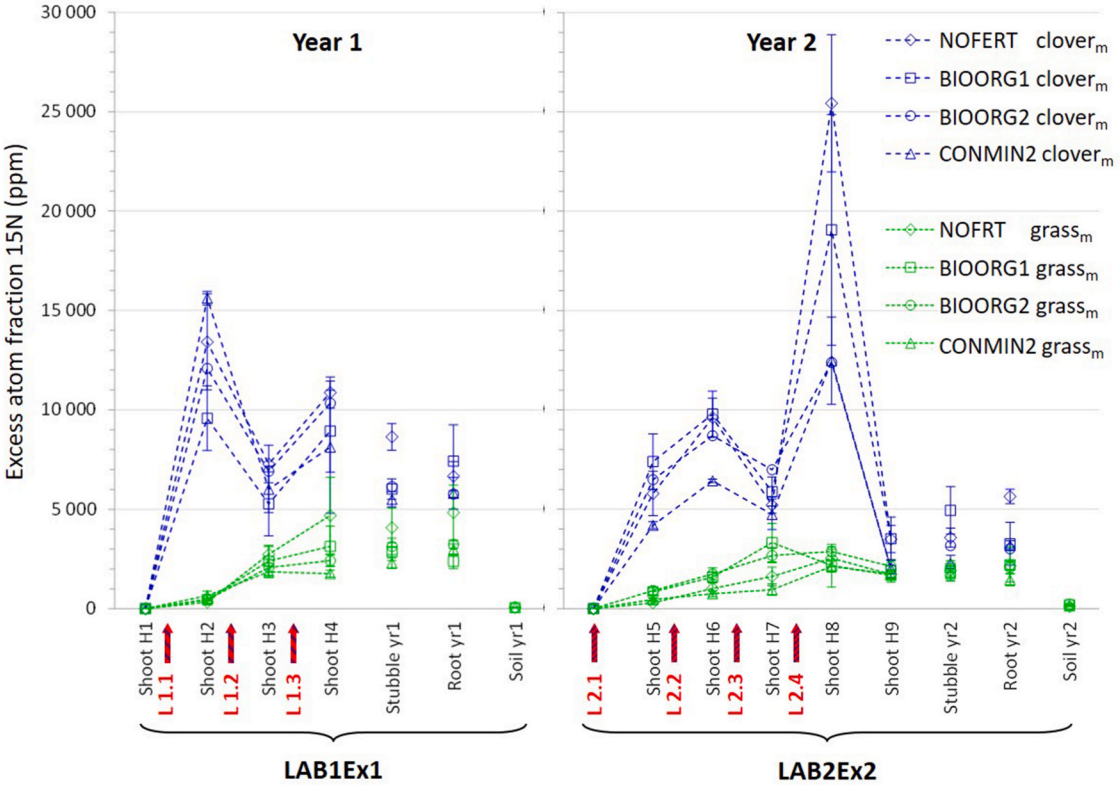

Fig. 4. Temporal development of the ^{15}N enrichment of ^{15}N labelled clover in mixture ($clover_m$) plant parts (roots as proxy for source 1), unlabelled associated grass plant parts (receiver), and the soil (0–0.25 m) from LAB microplots (basis for procedure LAB I, see Table 3).
Year 1data are from microplots LAB1Ex1; clover labelling events during year 1 are indicated as L1.1 to L1.3 ↑; roots and soils were excavated at the end of year 1: year 2 data are from microplots LAB2Ex2; clover labelling events during year 2 are indicated as L2.1 to L2.4 ↑ roots and soils were excavated at the end of year 2: error bars represent ± one *SEM*; for details of fertilisation treatments see Table 1; data are given in detail in Table S5.

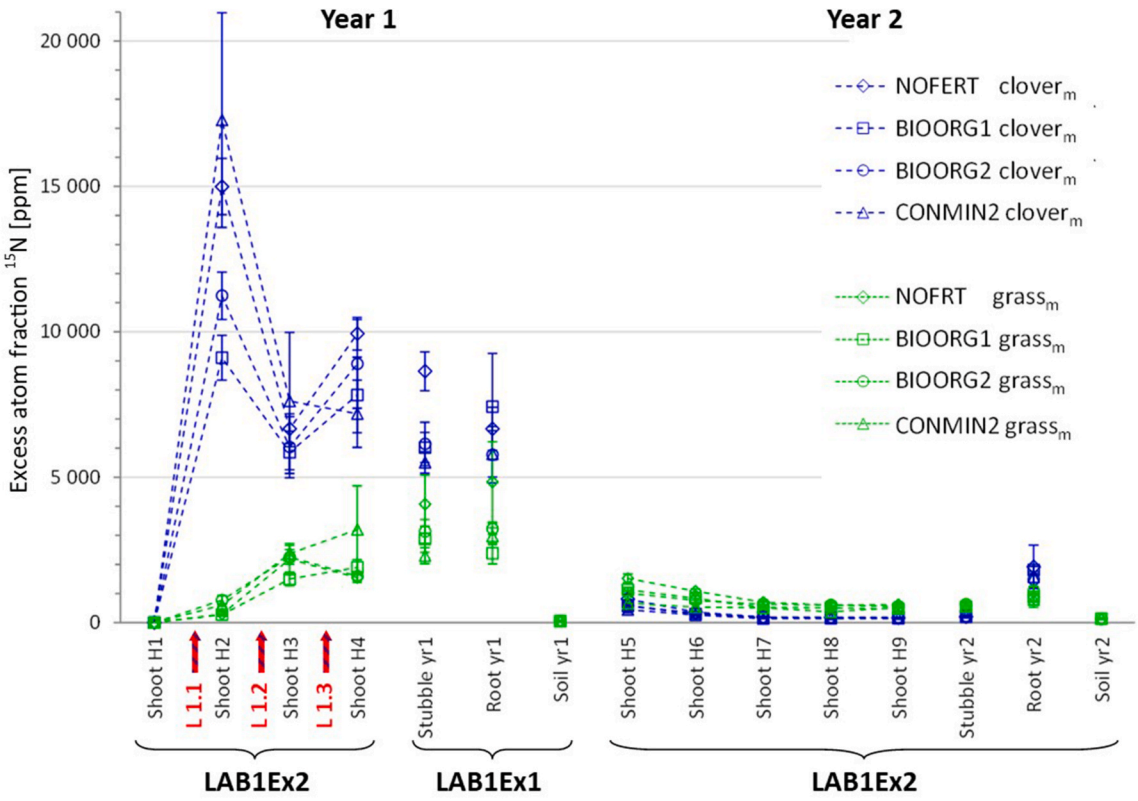

Fig. 5. Temporal development of the ^{15}N enrichment of ^{15}N labelled clover in mixture (clover$_m$) plant parts (roots as proxy for source 1), unlabelled associated grass plant parts (receiver), and the soil (0–0.25 m) from LAB microplots (basis for LAB II and LAB III, see Table 3).
Year 1 data are from microplots LAB1Ex2 (harvested shoots) and LAB1Ex1 (stubble, roots, and soil); clover labelling events during year 1 are indicated as L1.1 to L1.3 ↑, roots and soils were excavated at the end of year 1; year 2 data are from microplots LAB1Ex2; clover was not additionally labelled, roots and soils were excavated at the end of year 2; error bares represent ± one *SEM*; for details of fertilisation treatments see Table 1; data are given in detail in Table S5.

pure grass plant parts with the use of the δ^{15}N of the shoots of harvest 1 of grass in mixture (Table 2) and will discuss the underlying assumptions and the evolution of these δ^{15}N signatures hereafter. First, the fact that after harvest 1 the δ^{15}N of the grass in mixture decreased more strongly than that of the pure grass is evidence of N transfer from clover. The reasons for using the δ^{15}N of grass in mixture from harvest 1 were discussed in detail by Oberson et al. (2013). In brief, this approach used only one microplot to determine the δ^{15}N of the sources and of the

Table 5
Proportion of grass N transferred from clover in year 1 and year 2 determined by the^{15}N natural abundance (NA) and the^{15}N labelling (LAB) method, using different calculation (NA and LAB) and labelling (LAB) procedures. The NA procedures differed in which plant parts were analysed to represent the δ^{15}N signatures of N transferred from clover (source 1), plant available soil N (source 2), and grass in mixture (receiver) (see Table 2). The LAB procedures differed in clover labelling, use of microplots, and/or calculation protocols (see Table 3).

Method & procedure (M&P)	Proportion of grass N transferred from clover (PGNtfc) [a]									
	Year 1					Year 2				
	NOFERT	BIOORG1	BIOORG2	CONMIN2	M&P	NOFERT	BIOORG1	BIOORG2	CONMIN2	M&P
NA I	35% (3)	29% (4)	24% (4)	20% (1)	27% B	40% (4)	44% (4)	38% (3)	28% (2)	37% B
NA II	44% (4)	45% (4)	37% (3)	56% (2)	45% A	44% (4)	53% (4)	47% (3)	35% (2)	45% AB
NA III[b]	49% (4)	42% (4)	41% (4)	48% (3)	45% A	43% (4)	51% (4)	46% (4)	38% (3)	44% B
NA IV	53% (4)	43% (4)	46% (3)	49% (2)	48% A	45% (4)	55% (4)	45% (4)	38% (3)	46% AB
LAB I	59% (3)	50% (4)	56% (4)	39% (3)	51% A	42% (4)	59% (4)	68% (3)	45% (3)	54% A
LAB II	61% (4)	45% (4)	53% (4)	45% (3)	51% A	46% (4)	43% (4)	46% (4)	40% (3)	44% B
LAB III						25% (4)	23% (4)	26% (4)	21% (3)	24% C
Treatment	50%	42%	42%	42%		40% ab	47% a	45% a	34% b	
SEM/Mean					1.5% / 45%					1.4% / 42%
Treatment					n.s.					**
M&P					***					***
Treatment x M&P					n.s.					n.s.

For detailed explanations of fertilisation treatments see Table 1; two factorial mixed effect model (treatment x procedure + error [replication, row]); *n* of the treatment's mean in parentheses; n.s., **, and *** represent *p* values > 0.05 (not significant), <0.01, and <0.001, respectively; *t*-test LSD, α < 0.05; same letters indicate no significant difference between factors; lowercase letters refer to treatments and capital letters refer to methods & procedures.

[a] Data were centred log-ratio transformed.

[b] Method and procedure selected to quantify the amount of grass N transferred from clover and the amount of grass N derived from atmosphere via transfer of clover N (see Table 6).

Table 6
Amount of grass N transferred from clover (AGNtfc), proportion of clover N derived from atmosphere via symbiotic N_2 fixation (PCNdfa), and amount of grass N derived from atmosphere via transfer of symbiotically fixed clover nitrogen N (AGNdfa) for year 1 and year 2 under different fertilisation treatments.

	AGNtfc [g m^{-2}] [a]			PCNdfa [b]			AGNdfa [a] [g m^{-2}]		
	Year 1	Year 2	T	Year 1	Year 2	T	Year 1	Year 2	T
NOFERT	1.5[c]	3.0[b]	2.1	92%	94%	93% a	1.4	2.8	2.0 b
BIOORG1	1.9 [bc]	5.9[a]	3.4	89%	91%	90% a	1.7	5.3	3.0 a
BIOORG2	2.1 [bc]	6.3[a]	3.6	86%	90%	88% a	1.8	5.7	3.2 a
CONMIN2	4.9[a]	6.2[a]	5.5	69%	78%	74% b	3.3	4.8	4.0 a
Year	2.3	5.1		86% A	89% B		1.9 B	4.5 A	
SEM (*n*) mean			0.45 (30) 4.0			1.6% (30) 87%			0.4 (30) 3.4
Treatment (T)			***			***			**
Year (Y)			***			*			***
T x Y			*			n.s.			n.s.

For details of fertilisation treatments see Table 1; one factorial mixed effect model (treatment + error [replication, row]).
[a] Data were log transformed.
[b] Data were centred log ratio transformed.

receiver, thus reducing the effect of spatial variability. It assumed that the grass of harvest 1 had not yet received transferred clover N and that its $\delta^{15}N$ was representative of available soil N for the entire study period. While the first assumption is clearly supported by similar $\delta^{15}N$ of pure grass and grass in mixture of harvest 1 (Fig. 3), the second one is valid only if the decrease in the receiver's $\delta^{15}N$ is solely related to N transferred from clover and not due to a temporal decrease in the $\delta^{15}N$ of plant available soil N. The latter could be due to an increased input of a fertiliser with a low $\delta^{15}N$ signature such as a mineral N fertiliser or to a greater share of nitrate in the mineral N pool (Robinson, 2001). Since in our study NOFERT did not receive any fertiliser, the change in $\delta^{15}N$ due to fertilisation can be excluded. Because the amount of plant available soil N was lower under pure grass than under clover-grass mixtures (data not shown) due to lower fertiliser N requirements of clover-grass mixtures (Nyfeler et al., 2009, 2011), the $\delta^{15}N$ of N taken up by grass may have differed between pure grass and grass in mixture. Additionally, the pure grass N uptake always exceeded fertiliser N input (Table S1, Table 1). Thus, pure grass stands possibly utilized both a different N uptake pattern and different N dynamics than the clover-grass mixtures. The total N uptake of pure grass was 1.1 (CONMIN2) to 1.9 (NOFERT) times higher than that of grass in mixture (Table S1, $p = 0.01$), suggesting that pure grass may have taken up plant available soil N from spatially and temporally different pools than grass in mixture. Grass in mixture was shown to root more deeply than pure grass (Boller and Nösberger, 1988), and $\delta^{15}N$ signatures may change throughout the soil profile (Kramer et al., 2017). We found greater root dry matter of pure grass than of grass in mixture in the layer 0–0.3 m. The ratio of root dry matter of pure grass and grass in mixture was 1.7 at the end of year 1, significantly lower than the ratio of 2.9 at the end of year 2 (data not shown, $p = 0.002$). Both proxies for the $\delta^{15}N$ of plant available soil N had specific problems (pure grass was grown in spatially different microplots, possibly acquiring more plant available soil N from different soil layers; harvest 1 of grass in mixture was temporarily shifted from the following harvests of the receiver grass in mixture). However, they had comparable $\delta^{15}N$ values over time, except for NOFERT and CONMIN2 in year 1 (Table S4). Consequently, proxies had little impact on the resulting transfer values, except for NA I in year 1. Thus, the $\delta^{15}N$ of the shoots of harvest 1 of grass in mixture generally represent a valid reflection of the $\delta^{15}N$ of plant available soil N. Hence, our results confirm the approach used by Oberson et al. (2013). The determination of PGNtfc requires only the sampling of shoots from clover and grass in mixture, whereby the $\delta^{15}N$ of plant available soil N can be determined on the grass shoots of first harvest after establishment of the mixture. The subsequent decrease in $\delta^{15}N$ of the grass shoots can then be used to derive the transfer; i.e., grass presents a source $\delta^{15}N$ signature of plant available soil N in harvest 1 and becomes the receiver thereafter.

4.2. Nitogen-15 natural abundance method works under organic and mineral fertilisation

The NA method depends on distinct differences in $\delta^{15}N$ between the two N sources of the grass in mixture. The difference between the $\delta^{15}N$ of clover N and of plant available soil N (as reflected by its proxies) was at least 3.9‰ in all treatments except for CONMIN2, which usually differed by less than 2‰ (Fig. 3, Table S2 and Table S3). Still, the differences between the $\delta^{15}N$ of clover, which ranged from 0.0‰ to −1.2‰, and the $\delta^{15}N$ of the shoots of grass in mixture of harvest 1, which ranged from +1.2‰ to 5.1‰ (Tables S2 and S3), were significant under all treatments, with differences from 1.4‰ (CONMIN2) to nearly 6‰.

We expected these lower differences in $\delta^{15}N$ between clover and grass in mixture shoots of harvest 1 under CONMIN2 rather than under other treatments because of the low $\delta^{15}N$ value of applied mineral fertilisers, which was close to the $\delta^{15}N$ of atmospheric N_2 (Table 1). However, in an earlier study, the grass of harvest 1 growing in mixture under CONMIN2 had higher $\delta^{15}N$ values than in the present study, resulting in differences of more than 2‰ between the two sources (Oberson et al., 2013). This might have been due to the much higher grass proportion in the field plots (≈70%) studied by Oberson et al. (2013) compared to the microplots of our study (≈20%, see Table 3) resulting in nearly two times greater N uptake by second year grass in mixture (shoot N: 22.0 g m^{-2} [Oberson et al., 2013] compared to 11.3 g m^{-2} [Table S1]). Thus, the greater N demand of grass in mixture in the field plots combined with slightly lower fertiliser N input (compare Table 3 in Oberson et al., 2013 with Table 1) likely resulted in greater uptake of soil derived N, which has a higher $\delta^{15}N$ than the mineral N fertiliser (Table 1 and Oberson et al., 2013). Because leys are usually managed to achieve a botanical composition similar to that reported by Oberson et al. (2013), differences between $\delta^{15}N$ values of clover and grass from harvest 1 could be higher than differences found in the present study unless higher mineral N fertiliser doses were applied.

Clover-grass mixtures are usually fertilised at zero to moderate N levels, i.e. between 0 and 150 kg N ha^{-1} a^{-1} (Nyfeler et al., 2011) with higher N levels usually being only applied to pure grass stands. Nyfeler et al. (2011) showed that moderate N fertilisation only slightly decreased the clover proportion as well as the N fixation rate. Thus, at common N fertilisation of mixtures, the ^{15}N NA method should be applicable as demonstrated with our treatment CONMIN2.

4.3. Obtaining a constant ^{15}N enrichment of the root is challenging

The LAB method assumes a constant ^{15}N enrichment of the roots over time. However, this was not the case in the present study (Figs. 4 and 5) although the ^{15}N label input was adapted to the expected clover N uptake based on clover-grass mixtures previously cultivated in the DOK experiment (Oberson et al., 2013). Roots sampled after harvest 2

(following the first labelling of year 1) had EAF ^{15}N values from about 2200 ppm to 3150 ppm (Hammelehle et al., 2018). In contrast, the roots' ^{15}N enrichment in microplot LAB1Ex1 excavated at the end of year 1 (Fig. 1) was two to three times higher (ranging from 5113 ppm to 6697 ppm, depending on the treatment) and decreased to 2793 ppm to 5297 ppm until the end of year 2, since labelling was discontinued in year 2 (assuming the same enrichment of the roots at the end of year 1 in LAB1Ex2 as in LAB1Ex1, Fig. 5). To alleviate the impact of temporally variable ^{15}N enrichment, we compared three procedures for year 2. With LAB I, which uses the data from LAB2Ex2 shown in Fig. 4, labelling was performed during year 2 and roots were sampled at the end of that year. With LAB II, which uses the data of LAB1Ex2 shown in Fig. 5, labelling was performed during year 1 only, but roots were sampled at the end of year 2. Since the roots harvested at the end of year 1 (LAB1Ex1) were significantly more highly enriched in ^{15}N compared to the roots harvested at the end of year 2 (LAB1Ex2), we implemented a third procedure using the geometric mean of the ^{15}N enrichment of roots excavated at the end of year 1 from microplot LAB1Ex1 and at the end of year 2 from LAB1Ex2 (LAB III, Equation (12)). We expected that the geometric mean would represent the average ^{15}N enrichment of the second year more correctly than the EAF ^{15}N determined from the roots excavated at the end of year 2 from LAB1Ex2. However, we do not know the time course of the roots' ^{15}N enrichment for that period (modelling the time course gave similar results as LAB III [data not shown]). Thus, LAB II assumes, as does LAB I, that the EAF ^{15}N of a root sampled at the end of the year of interest represents the EAF ^{15}N of the entire year. However, this assumption was likely not met by what could be assumed from the temporally great variability of the shoots' EAF ^{15}N values (Figs. 4 and 5). The comparison of results obtained with LAB II and LAB III exemplifies the impact of different root EAF ^{15}N values on the PGNtfc, which differed by a factor of almost 2. When using LAB for determining PGNtfc, e.g., in situations where the differences in $\delta^{15}N$ between the two sources might be too small, we therefore recommend determining the roots' ^{15}N enrichment more frequently to enhance the temporal resolution and to calculate the PGNtfc for each harvest, similar to the NA procedures (Equation (3), Equations S(1)–(3)). This might result in less variability between the LAB procedures.

In our study, NA procedures provided more robust results than LAB in year 2 when different NA procedures resulted in less variable PGNtfc than LAB. NA III fitted best with the average obtained from all methods for all treatments and the average over all treatments (Table 5, Fig. S1). Moreover, NA III required only sampling of shoots from the mixture. Therefore, we used it for the quantification of AGNtfc and AGNdfa.

4.4. Quantification of N transfer and N fixation

According to the PGNtfc obtained with NA III, more than 40% of grass N was transferred from associated clover in year 1 irrespective of N fertilisation (NA III, Table 5). This proportion was similar to that of year 2. The clover proportion of CONMIN2 remained at the same level in both years but decreased somewhat in the other treatments, although it remained at more than 80% (Table 4). Compared to literature PGNtfc data, which ranged from 0% to 60% (Chalk et al., 2014; Peoples et al., 2015), our results were in the medium range. Experiments have differed in fertiliser N inputs, from low (Boller and Nösberger, 1988; Dahlin and Stenberg, 2010; Nyfeler et al., 2011; Oberson et al., 2013) to moderate (Boller and Nösberger, 1987; Nyfeler et al., 2011; Dahlin and Stenberg, 2010; Oberson et al., 2013), to high (Nyfeler et al., 2011), and both in clover proportions in the sward and in applied methods; NA (Oberson et al., 2013; Schipanski and Drinkwater, 2012), and LAB (Boller and Nösberger, 1987, 1988; Dahlin and Stenberg, 2010; Nyfeler et al., 2011; Rasmussen et al., 2007). Overall, the literature suggests a relation between increasing fertiliser N input and slightly decreasing PGNtfc together with decreasing clover proportions. This is supported by our data, though at relatively high clover proportions.

The AGNtfc ranged from 1.5 to 4.9 g m^{-2} in year 1 and from 3.0 to 6.3 g m^{-2} in year 2 (Table 6). This was lowest under NOFERT and highest in CONMIN2 in year 1, while all fertilised treatments had similar amounts in year 2. The amount is at a medium level compared to the literature, which ranges from 0 to 4.2 g m^{-2} a^{-1} using the ^{15}N dilution method (Boller and Nösberger, 1987; Dahlin and Stenberg, 2010) and 4–11 g m^{-2} a^{-1} using the NA method (Dahlin and Stenberg, 2010; Oberson et al., 2013). Compared to Oberson et al. (2013), our lower values can be explained by lower grass proportions in this study.

The proportions of fixed N in clover of the zero and low N fertilised treatments were, at about 90%, generally high (PCNdfa in Table 5) and at an upper level of the reported literature data, which ranges from 58% to 99% (Chalk et al., 2016). The PCNdfa of the moderately N fertilised treatment CONMIN2 was significantly lower but increased from 69% in year 1% to 78% in year 2. Nitrogen fixation rates decrease even more at high N levels as well as at high clover proportions since legumes downregulate the symbiotic N_2 fixation with closing gaps between their N demand and the N supply (Hartwig, 1998, Soussana und Tallec, 2010).

Considering the proportions of fixed N in the clover source, grass in mixture contained 1.4–4.8 g m^{-2} of AGNdfa in shoots harvested during one year. This amount represents an important input of symbiotically fixed N in addition to the amount of clover N derived from the atmosphere. However, the share of AGNdfa to the total amount of N derived from the atmosphere in the shoots and roots of the mixture (amount of clover N derived from the atmosphere and AGNdfa) was low, 5%–7%, due to a much lower N uptake by grass than by clover (Fig. 2). This share would increase with a higher grass proportion in the mixture, as is usually found in experimental fields (Nyfeler et al., 2011; Oberson et al., 2013) and farmers' fields (Suter et al., 2021).

5. Conclusions

Our study demonstrates that $\delta^{15}N$ values of the different N sources in grass-clover leys are distinct enough to determine the N transfer from clover to grass with the NA method across different fertilisation treatments covering a wide range of N fertilisers, application rates, and fertiliser $\delta^{15}N$ signatures. Moreover, proportions of N transfer obtained with different NA procedures varied less than those obtained with different LAB procedures. In the NA procedures, $\delta^{15}N$ signatures of different proxies representing those of the sources, i.e., clover N and plant available soil N, and the receiver, i.e., grass in mixture, had little impact on the proportion of N transfer. Thus, the NA method is robust. The procedure that agreed best with the average results over all NA and LAB methods was the NA procedure that required only the ^{15}N signatures of the shoots from clover and grass grown in mixture (NA III). Thereby, the ^{15}N signature of plant available soil N was represented by that of the grass shoots of harvest 1, while the declining ^{15}N signatures of later harvests of grass shoots demonstrated that they were receivers. For LAB, given the sharp changes in ^{15}N excess of clover shoots over short time intervals due to leaf labelling, a constant ^{15}N enrichment of clover roots over time seems difficult to attain. If LAB were to be applied, then clover root sampling at each harvest is recommended to determine the enrichment of the clover roots at the same time as the enrichment of the harvested shoots. In both years, more than 40% of grass N was transferred from clover. This resulted in 1.5–6.3 g N m^{-2} of clover N annually transferred to grass depending on the fertilisation history of the cropping system. With 74% to 93% of clover N having been symbiotically fixed, this transfer presented a significant input of atmospheric N.

CRediT authorship contribution statement

Andreas Hammelehle: Conceptualization, Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft.

Jochen Mayer: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

Andreas Lüscher: Conceptualization, Formal analysis, Methodology, Supervision, Validation, Writing – review & editing, Funding acquisition.

Astrid Oberson: Conceptualization, Formal analysis, Funding acquisition, Methodology, Validation, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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