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REGULAR ARTICLE

Above- and belowground nitrogen distribution of a red clover-perennial ryegrass sward along a soil nutrient availability gradient established by organic and conventional cropping systems

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Abstract
Aims Belowground legume nitrogen (N) composed of roots and rhizodeposition is an important N input to soils, but published data of belowground N vary broadly, probably due to extrapolation from short-term experiments and dissimilar growing conditions. We quantified belowground N inputs of red clover (*Trifolium pratense* L.) during two consecutive years in a clover-grass sward along a soil nutrient availability gradient.

Methods We established a red clover-perennial ryegrass (*Lolium perenne* L.) model sward in microplots located in field plots of the DOK experiment, which has a 33-year history of organic and conventional cropping, resulting in a soil nutrient availability gradient. Four treatments were examined: the zero fertilisation control, bio-organic with half and full dose manure application, and the conventional system with mineral fertilisation at full dose. We studied the development of clover aboveground and belowground N using multiple pulse ^{15}N urea leaf labelling.
Results Belowground clover N increased over time and with rising nutrient availability and was proportional to aboveground clover N at all times. Belowground clover N amounted to 40% of aboveground clover N during two consecutive years, irrespective of the nutrient availability status. Belowground clover N development was initially dominated by fast root growth, followed by enhanced root turnover during the second year. Potassium availability limited clover growth and total N accumulation in treatments with low nutrient availability.
Conclusions Belowground red clover N inputs could be estimated from aboveground N by a constant factor of 0.4, regardless of the nutrient availability and cultivation time. Root turnover led to a distinct absolute increase of N rhizodeposition over time. Hence, N rhizodeposition, with an 80% share of belowground N, was the predominant N pool at the end of the second year.

Keywords Rhizodeposition · ^{15}N leaf labelling · Cropping systems · Belowground to aboveground N ratio · Nutrient availability

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Abbreviations

AGN	Aboveground N
BGN	Belowground N, comprising physically recoverable root N at the time of excavation plus NdfR
BIOORG1	Bio-organic treatment of the DOK experiment with half dose fertilisation
BIOORG2	Bio-organic treatment of the DOK experiment with full dose fertilisation
CONMIN2	Conventional treatment of the DOK experiment with full dose sole mineral fertilisation
CFE	Chloroform fumigation extraction
DOK	Long-term experiment comparing Bio-**D**ynamic, Bio-**O**rganic, and conventional (**K**) cropping systems
EAF	Excess atom fraction
LMP(t)	Labelled microplot, delimiting the ^{15}N labelled plant-soil system (excavated after t months of sward cultivation)
NdfR	Nitrogen derived from rhizodeposition
NOFERT	Unfertilised control treatment of the DOK experiment
RMP(t)	Reference microplot, delimiting the unlabelled plant-soil system (excavated after t months of sward cultivation)
t	Time from planting of red clover and perennial ryegrass until microplot excavation in months

Introduction

Legume-rhizobia symbioses provide annually around 40 Tg reactive nitrogen (N) to agro ecosystems worldwide (Herridge et al. 2008), which compares to one third of the amount of technically bound N from the Haber-Bosch process (121 Tg, Galloway et al. 2008). About 40% of legume-rhizobia fixed N is contributed by fodder legumes, which acquire on average 70% of their N by symbiotic N_2 fixation (Herridge et al. 2008). The proportion of N derived from symbiotic N_2 fixation of red clover (*Trifolium pratense* L.) generally exceeds 80% if grown in mixture with grass and fertilised moderately with mineral N (Boller and Nösberger 1987; Nesheim and Øyen 1994; Huss-Danell et al. 2007; Oberson et al. 2013).

Perennial ryegrass (*Lolium perenne* L.), a common mixture partner in clover-grass swards, is highly competitive for soil mineral N (Nyfeler et al. 2011), due to the greater root length and root surface area compared with red clover (Mengel and Steffens 1985). Several studies have shown that grasses cultivated in clover-grass swards can obtain 30% to 60% of their N from clover (Boller and Nösberger 1987; Dahlin and Stenberg 2010a; Oberson et al. 2013; Schipanski and Drinkwater 2012). This significant N transfer is probably due to the regular cutting of the sward, which might induce root turnover of forage plants (Hamilton et al. 2008), followed by microbial incorporation and mineralisation (Haystead and Marriott 1979). Indeed, Sierra et al. (2007) and Trannin et al. (2000) observed cutting-induced N transfer rates from legume trees to grasses, which strongly increased after pruning. The cutting-induced N transfer found by Sierra et al. (2007) was 12 times higher than the N transfer via exudation. This observation suggests that cutting triggers root decay followed by a fast turnover of the root debris, as assumed by several authors (Fustec et al. 2010; Hamilton et al. 2008; Haystead and Marriott 1979; Ta and Faris 1987; Thilakarathna et al. 2016).

Root debris N is part of N derived from rhizodeposition (NdfR), comprising all kinds of compounds lost from living plant roots, including exuded organic compounds, ions, and volatile compounds (Uren 2007). For operational reasons, Mayer et al. (2003) defined NdfR as root derived N remaining in the soil after sorting out visible roots. Hence, belowground N (BGN) comprises root N and NdfR. As much as 70% of total N in fodder legumes was reported to be BGN (Herridge et al. 2008), which thus plays an important role in the N cycle of clover-grass mixtures. However, the published data basis is small and varies strongly, from 20% to 30% (Dahlin and Mårtensson 2008; Gylfadóttir et al. 2007), to 50% (Dahlin and Stenberg 2010b), and up to 70% (McNeill et al. 1997). While Herridge et al. (2008) attributed the variation to effects of species, soil, and climate on the partitioning of N within the plant, management (cutting vs. grazing, intensity and frequency; Dahlin and Mårtensson 2008; Dahlin and Stenberg 2010a, b) and biotic as well as abiotic stress (e.g. pests, diseases, temperature, water; Haase et al. 2007) also might affect the partitioning. Beside these effects, a strong variation might come from extrapolation to full years of short-lived examination periods and dissimilar growing conditions.

Nitrogen derived from rhizodeposition is usually determined using ^{15}N stable isotope labelling of the root N, with the isotope being applied via stems or leaves (Fustec et al. 2010; Wichern et al. 2008). The percentage of NdfR can then be calculated from the ^{15}N enrichment of the root-free soil assuming that NdfR and root N have the same ^{15}N isotopic composition (Janzen and Bruinsma 1989).

In the DOK long-term experiment, Oberson et al. (2013) observed that low nutrient-supplied clover-grass swards were limited in potassium (K) and possibly co-limited in phosphorus (P). Nutrient limitations generally reduce aboveground growth, but may affect the shoot to root ratio in different manners. Potassium limitation was found to extend the shoot to root ratio of grain legumes, due to an inhibition of photosynthate-translocation to the root (Cakmak et al. 1994). Low N and P supplies, however, generally result in lower shoot to root ratios in grain legumes (Cakmak et al. 1994), leguminous (Almeida et al. 1999; Hill et al. 2006), and gramineous pasture plants (Hill et al. 2006) by increasing the absorptive root surface at the expense of aboveground biomass (Hill et al. 2006). The active release of exudates to mobilise nutrients from the soil represents a further plant strategy. Especially carboxylates and phosphatases are exuded to mobilise P (Neumann and Römheld 2012).

While red clover aboveground N (AGN) in response to nutrient availability gradients has been studied extensively (e.g. Boller and Nösberger 1987; Davis 1991; Nyfeler et al. 2011; Oberson et al. 2013; Tucker and Smith 1952), little is known about the effect of the nutrient availability status on the relative size of red clover AGN, BGN, NdfR, and root N. To our knowledge, the effect of N, P, and K availability on the red clover BGN to AGN ratio has not yet been examined under a well-established nutrient availability gradient with identical climatic and pedologic conditions. Furthermore, red clover BGN was usually determined at one point of time (Dahlin and Stenberg 2010b; Høgh-Jensen and Schjoerring 2001; Huss-Danell et al. 2007) and was only once determined throughout two consecutive years (Høgh-Jensen and Schjoerring 2001). Therefore, little is known about the development of red clover root N and NdfR during a two-year cultivation period.

The objectives of our study were i) to investigate the development of red clover BGN and its components root N and NdfR in relation to red clover AGN in a red clover-perennial ryegrass model sward during two consecutive years and ii) to study the effects of different nutrient availabilities in organic and conventional cropping systems on the relative sizes of red clover AGN, BGN, root N, and NdfR. Root N and AGN development of the grass-partner in the sward were also investigated.

We expected a first phase with fast red clover root growth during the first months of establishment and a second phase with a steady root turnover. In this second phase, red clover BGN was expected to further increase through a continued release of rhizodeposits, leading to an increasing proportion of NdfR to BGN over time. With decreasing nutrient availability, we expected enhanced red clover root growth and rhizodeposition relative to red clover aboveground biomass and AGN development.

Material and methods

The DOK long-term field experiment (Maeder et al. 2002), which compares bio-**D**ynamic, bio-**O**rganic, and conventional (**K**onventionell) cropping systems (DOK), was used as an experimental platform. A microplot study with a model red clover-perennial ryegrass sward was carried out in the regular clover-grass sward of the DOK crop rotation in 2011 and 2012.

The DOK experiment was established in 1978 in Therwil (near Basle, Switzerland; 307 m above sea level; 7°33′ E, 47°30′ N) at a site with a haplic Luvisol developed on loess (Maeder et al. 2002). During the two consecutive years of study, the mean annual temperature was 11.6 °C in 2011 and 10.9 °C in 2012 and the mean annual precipitation was 688 mm in 2011 and 1048 mm in 2012 (Fig. S1 and Fig. S2). The management and the experimental design were described thoroughly by Mayer et al. (2015). From 2006 until 2012, the seven year crop rotation comprised silage maize (*Zea mays* L.), winter wheat I (*Triticum aestivum* L.), soybean (*Glycine max* (L.) Merr.), potato (*Solanum tuberosum* L.), winter wheat II, and two full years of clover-grass sward (2011 and 2012). Treatments differ in amount and form of applied fertilisers and in crop protection. The following four DOK treatments, characterised by increasing nutrient availability (Table 1), were examined in the present study: the unfertilised control (NOFERT), two bio-organic treatments receiving either manure at full dose at fertilisation level 2 (BIOORG2) or at half

Table 1 Soil properties of the examined treatments of the DOK experiment and average annual nutrient inputs to the model red clover-perennial ryegrass swards grown in the microplots between 2010 and 2012 and to the swards grown in the field plots between 1978 and 2012 (numbers in parentheses)

Treatment	Soil properties [mg kg^{-1}] [1]				SOC : SON	Average annual input in microplots (field plots) [g m^{-2} a^{-1}] [1]									
	SON	Mineral N	Available P	Available K		Total N		Mineral N		Phosphorus		Potassium		Total C	
NOFERT	1330	14.2	0.3 ^{c}	2.7 ^{c}	8.6 ^{b}	0	(0)	0	(0)	0	(0)	0	(0)	0	(0)
BIOORG1 [2]	1367	17.6	0.5 ^{b}	7.3 ^{b}	8.8 ^{a}	1.9	(4.5)	1.2	(1.5)	0.3	(1.2)	8.7	(8.4)	18.7	(58.5)
BIOORG2 [2]	1468	21.7	0.9 ^{a}	13.7 ^{a}	9.0 ^{a}	3.9	(9.0)	2.3	(2.9)	0.5	(2.5)	17.4	(16.7)	37.4	(116.9)
CONMIN2 [3]	1365	16.7	1.2 ^{a}	11.5 ^{a}	8.9 ^{a}	14.0	(12.2)	14.0	(12.2)	2.0	(3.8)	16.0	(24.8)	0	(0)
SEM p	34 $^{n.s.}$	1.1 $^{n.s}$	0.1 ****	1.2 ****	0.06 *	–	(–)	–	(–)	–	(–)	–	(–)	–	(–)

ANOVA: mean of $n = 4$ (CONMIN2: $n = 3$); n.s. not significant, *, **, and *** significant at $p < 0.05$, 0.01, and 0.001, respectively; same letters indicate no significant difference between treatments; SON : SOC: ratio between soil organic carben and soil organic nitrogen

[1] Data were centred log-ratio transformed

[2] Fertiliser applied as farmyard manure

[3] Fertiliser applied as calcium ammonium nitrate, triple superphosphate, and potassium chloride

dose at fertilisation level 1 (BIOORG1), and a treatment representing a stockless conventional cropping system receiving only mineral fertiliser at full dose (CONMIN2) according to Swiss fertilisation guidelines (Flisch et al. 2009).

Manure application in the two organic treatments corresponds to a phosphorous (P) amount of 1.4 livestock units or 22 kg P ha^{-1} year^{-1} at level 2 and 50% of that at level 1. Soil properties, available soil nutrient contents (N, P, K), and nutrient inputs to the investigated swards are given in Table 1. The clover-grass swards received no pest or disease control agents. Details of the sward management are presented in Oberson et al. (2013). Mean fertiliser nutrient applications in 2011 and 2012 differed from the long-term applications (Table 1), due to a variation in nutrient contents of manure and an adaptation of the fertilisation to recent fertilisation guidelines. Overall, short- and long-term management with different amounts of fertiliser application led to a differentiation in nutrient availability of DOK treatments in soils of the swards in the order NOFERT < BIOORG1 < BIOORG2 < CONMIN2 (Table 1).

Microplot study

Per field plot of the examined treatments (4 treatments × 4 replications = 16 field plots), five microplots were installed by inserting a PVC tube (0.375 m inner-diameter corresponding to an area of 0.11 m^2; 0.3 m height) to a depth of 0.25 m into the undisturbed soil before sowing the clover-grass sward (Swiss standard mixture SM 330, Suter et al. 2012) in August 2010. Microplots were arranged in a line between the border and central area of the field plot. In contrast to the field plots, microplots remained uncultivated until spring 2011. On February 8, 2011, red clover (*Trifolium pratense* L., cv. Dafila) and perennial ryegrass (*Lolium perenne* L., cv. Lacerta) were seeded into pots containing soil of the future field plots, were pre-cropped in the greenhouse, and afterwards cold hardened in a cold frame outside the greenhouse. On March 29, 2011, microplots were each planted with 11 clover and 20 grass seedlings corresponding to an overall density of 300 plants m^{-2} (Fig. 1).

To determine red clover NdfR, clover plants were ^{15}N multiple-pulse labelled in three out of five microplots (LMP, Fig. 1). The remaining two microplots were kept unlabelled and served as reference microplots (RMP, Fig. 1) to determine the ^{15}N natural abundance background of the plot. One of each LMP was excavated after 4, 8, and 19 months of clover-grass sward cultivation, and one of each RMP was excavated after 8 and 19 months of cultivation. In total, the design comprised 48 LMP (4 treatments × 4 replicated field plots × 3 LMP per field plot) and 32 RMP (4 treatments × 4 replicated field plots × 2 RMP per plot). To avoid contamination of RMP microplots, LMP and RMP microplots were separated in two sub-units per

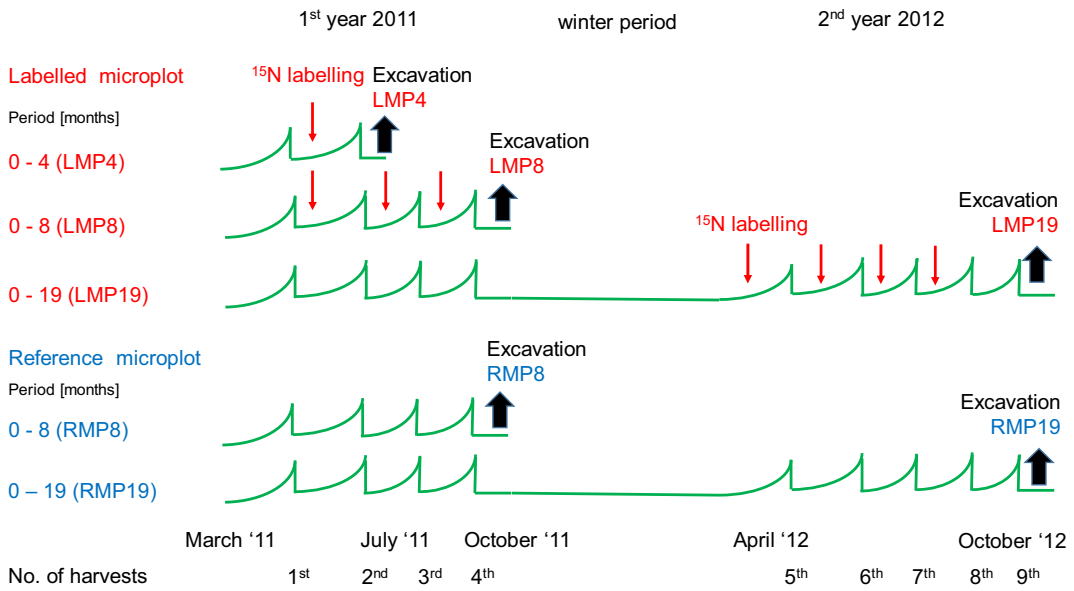

Fig. 1 Setup of the microplot-study with timeline of red clover plant labelling in labelled microplots (LMP), harvesting and re-growth (green / grey line) in LMP and in non ^{15}N labelled reference microplots (RMP), and excavation of LMP and RMP for belowground N determination. LMP4: 1st to 2nd harvest; 1st labelling before 2nd harvest; excavation at 4 months, after the 2nd harvest. LMP8: 1st to 4th harvest; labelling before 2nd, 3rd, and 4th harvest; excavation at 8 months, after the 4th harvest. LMP19: 1st to 9th harvest; labelling before 5th, 6th, 7th, and 8th harvest; excavation at 19 months, after the 9th harvest. RMP8: 1st to 4th harvest; excavation at 8 months. RMP19: 1st to 9th harvest; excavation at 19 months

field plot and were randomised within the respective sub-block.

Multiple ^{15}N pulse labelling

While all eleven clover plants per LMP were labelled by ^{15}N enriched urea solution, with the urea having an atom fraction ^{15}N of 990,000 ppm (ReseaChem, Switzerland), perennial ryegrass was not fed with ^{15}N (Fig. 1). To achieve a uniform ^{15}N root enrichment targeted at 5000 ppm excess atom fraction (EAF) ^{15}N, volume and urea concentration of the solution applied per plant ranged from 0.5 to 1.5 ml and from 0.3% to 0.5% (*w/v*), respectively, depending on the predicted N uptake as related to the treatment. The prediction was derived from data of Oberson et al. (2013). The label was generally applied about two weeks after the preceding sward harvest at one single leaf per clover plant and labelling event (Fig. 1) using the method according to Ledgard et al. (1985). The method was modified by squashing the trifoliate clover-leaf manually to damage the cuticle, thus facilitating uptake of the labelling solution. The squashed leaf was then inserted into a 2 ml vial. After pipetting the labelling solution, the vial was sealed airtight with Terostat X (Henkel, Germany) to prevent losses of labelling solution. Generally, the labelling solution was completely absorbed by the plant within 24 h. Vials together with the inserted leaves were removed not later than 72 h after starting the labelling. However, if the solution uptake was incomplete, which was the case at less than 10%, the remaining volume was recorded to enable ^{15}N recovery calculations (cf. Eq. 13). Leaf litter was collected from the soil surface once per week to prevent decomposition of labelled aboveground litter and, hence, tracer translocation to the soil.

The first LMP (LMP4) was labelled once in 2011, three weeks before the 2nd harvest, and was excavated after the 2nd harvest in July 2011, 4 months after planting. The second LMP (LMP8) was labelled three times in 2011, before the 2nd, 3rd, and 4th harvest (Fig. 1), and was excavated after the 4th harvest in October 2011, 8 months after planting. The third LMP (LMP19) was kept unlabelled in 2011, was labelled four times in 2012, before the 5th, 6th, 7th, and 8th harvest, and was excavated after the 9th harvest in October 2012, 19 months after planting (Fig. 1). The two RMP were kept unlabelled and were excavated after 8 and 19 months, respectively. The entire amount of red clover NdfR accumulated in the soil

at 19 months was determined by summing NdfR determined from LMP8 in 2011 and NdfR determined from LMP19 in 2012 (cf. Fig. 1, Eq. 8).

Sampling and processing of plant and soil samples

We quantified clover and grass total N, AGN, root N, and, additionally for clover, NdfR. To investigate the fate of NdfR in specific soil N pools (Hammelehle et al. unpublished), the soil was split in specific soil N pools using a sequential extraction. Nitrogen derived from rhizodeposition was subsequently determined for each single soil N pool (cf. Eq. 6) and summed up.

The sward's aboveground biomass growing in the microplots was harvested four times in 2011 (1st to 4th harvest) and five times in 2012 (5th to 9th harvest, cf. Fig. 1). Clover and grass plants were harvested separately by cutting them 0.05 m above the ground using manual garden shears. Before excavating the microplots, clover and grass stubble were separately cut off at the soil surface. Aboveground clover and grass samples were stored in cooling boxes for a maximum of 12 h until drying at 60 °C for 72 h. Dried samples were ground with a centrifuge mill (Retsch GmbH, Germany) to a size < 0.08 mm.

The microplot soil was split into six equal wedges each excavated to a depth of 0.25 m and weighed separately. Three out of six wedges were combined to a composite sample and used for further processing. Additionally, composite subsoil samples consisting of five cores each from 0.25 to 0.6 m taken with a gouge auger (diameter 0.05 m, Eijkelkamp, Netherlands) were excavated at 8 and 19 months from LMP8 and LMP19, respectively (Fig. 1). Soils were stored for between one and four weeks at 4 °C until further processing.

Field moist soil samples were crumbled and separated manually from visible macro roots. The water content was determined from subsamples of crumbled soil dried at 105 °C until constant weight. After macro root separation, the field moist crumbled soil sample was sieved at 3 mm. Roots (> 3 mm) remaining on the sieve were collected. Macro roots and remaining roots from sieving were divided visually into nodulated clover roots, grass roots, and non-classifiable roots (mixture of clover and grass roots). Subsequently, roots were thoroughly cleaned using deionised water.

According to the procedure used by Mayer et al. (2003), non-classifiable rootlets < 3 mm were quantified using three aliquots of 150 g field moist soil (sieved at 3 mm) by shaking each aliquot overhead together with 500 ml 0.05 *M* K_2SO_4 at 39 r min^{-1} for 0.5 h. Subsequently, the soil-extract suspension was poured through a 0.5 mm sieve. Remaining non-classifiable rootlets were thoroughly cleaned with deionised water.

In the pre-extraction, the collected soil-K_2SO_4 suspension from the rootlet separation was then vacuum filtrated through a membrane filter (porafil® CA, Machery-Nagel, Germany) resulting in the root-free soil residue and the pre-extract. The root-free soil residue was further processed by chloroform fumigation at 20 °C for 24 h (Brookes et al. 1985) using 50 ml ethanol-free chloroform (Lichrosolv®, Merck Nr. 1.02444.1000, Germany). Subsequently, the fumigated soil was extracted with 300 ml 0.05 *M* K_2SO_4 (chloroform fumigation extraction, Wichern et al. 2007) and finally filtrated with a paper filter (MN 640d, Marcherey-Nagel, Germany) to separate the root-free soil from the extract containing microbial biomass N (Hammelehle et al. unpublished). Pre-extracts, comprising dissolved mineral N and organic N, and extracts from the chloroform fumigation extraction (CFE), containing microbial N, were processed by oxidation according to Cabrera and Beare (1993) and subsequently by NH_3 diffusion to quartz filter disks according to Mayer et al. (2003).

The total N concentration and the isotopic ratio ^{15}N to ^{14}N of plant samples, soil samples, and diffusion quartz-filter discs were determined 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., USA). Dissolved N in extracts was analysed with a TOC/TNb analyser (DIMA-TOC 100, Dimatec, Germany). Phosphorus and K concentrations of harvested clover and grass biomass were analysed per single harvest. For this purpose, plant samples were incinerated at 450 °C and ashes were solubilized with 6 *M* HCl. Afterwards, diluted filtrates were analysed by inductively coupled plasma atomic-emission spectroscopy using a Vista Pro ICP-OES (Varian, Agilent, USA) for P and K (Agroscope 1996).

To elucidate potential nutrient limitations of clover and grass during different development phases (0–4 months, 4–8 months, and 8–19 months) and taking into account the amounts of harvested biomass, weighted means of N, P, and K concentrations were calculated for the 1st to 2nd harvest (0 to 4 months), the 3rd to 4th harvest (5 to 8 months), and the 5th to 9th harvest (9 to 19 months).

Calculations

Cumulative AGN of red clover and perennial ryegrass [g m^{-2}] at the time *t* in months was calculated according to

$$\mathrm{AGN(t)}\ \left[\mathrm{g\ m^{-2}}\right] = \mathrm{stubble(t)N}\ \left[\mathrm{g\ m^{-2}}\right] + \sum_{i=1}^{n}\mathrm{harvest_i\ N}\ \left[\mathrm{g\ m^{-2}}\right] \quad (1)$$

where stubble(t) N corresponds to the standing stubble biomass N [g m^{-2}] at the time *t* when LMPt was excavated, and *n* corresponds to the number of harvests taken until the time *t* (cf. Fig. 1). Individual ^{15}N fed leaves together with their associated stalks were removed and not included in the analyses and calculations.

Soil total N [g m^{-2}] at the time *t*, when LMPt was excavated, was obtained by summing the N amounts of the soil N pools obtained from the pre-extraction and CFE extraction of the respective time:

$$\mathrm{Soil\ total\ N(t)}\left[\mathrm{g\ m^{-2}}\right] = \mathrm{N_{pre\text{-}extract}(t)}\left[\mathrm{g\ m^{-2}}\right] + \mathrm{N_{CFE-extract}(t)}\left[\mathrm{g\ m^{-2}}\right] + \mathrm{N_{root-free\ fumigated\ soil\ residue}(t)}\left[\mathrm{g\ m^{-2}}\right]. \quad (2)$$

The ^{15}N isotopic abundance of plant and soil samples is expressed as atom fraction ^{15}N [ppm (or μmol mol^{-1})] according to Coplen (2011):

$$\mathrm{Atom\ fraction\ ^{15}N\ [ppm]} = \frac{^{15}\mathrm{N}}{^{14}\mathrm{N} + {}^{15}\mathrm{N}} \times 1,000,000 \quad (3)$$

where ^{15}N or ^{14}N is the N amount of the respective isotope.

Excess atom fraction ^{15}N [ppm] of soil and plant samples was determined by differences in the atom fraction between LMPt samples and RMPt samples of the respective sample type and time (Jensen 1996):

$$\mathrm{EAF\ ^{15}N(t)} = \mathrm{atom\ fraction\ ^{15}N_{\ sample\ LMPt}} - \mathrm{atom\ fraction\ ^{15}N_{\ sample\ RMPt}} \quad (4)$$

Excess atom fraction ^{15}N of total soil N [ppm] was calculated as weighted mean of EAF ^{15}N of pre-extracts, CFE extracts, and fumigated soil residues according to

$$\mathrm{EAF\ ^{15}N_{\ soil\ total\ N}} = \frac{\mathrm{EAF\ ^{15}N_{\ pre-extract}} \times \mathrm{N_{pre-extract}}\left[\mathrm{g\ m^{-2}}\right] + \mathrm{EAF\ ^{15}N_{\ CFE-extract}} \times \mathrm{N_{CFE-extract}}\left[\mathrm{g\ m^{-2}}\right] + \mathrm{EAF\ ^{15}N_{\ root-free\ fumigated\ soil\ residue}} \times \mathrm{N_{root-free\ fumigated\ soil\ residue}}\left[\mathrm{g\ m^{-2}}\right]}{\mathrm{N_{pre-extract}}\left[\mathrm{g\ m^{-2}}\right] + \mathrm{N_{CFE-extract}}\left[\mathrm{g\ m^{-2}}\right] + \mathrm{N_{root-free\ fumigated\ soil\ residue}}\left[\mathrm{g\ m^{-2}}\right]}. \quad (5)$$

The proportion of red clover NdfR accumulated in the soil of the respective microplot LMPt until the time *t* of excavation was calculated according to Janzen and Bruinsma (1989):

$$\mathrm{Proportion\ of\ clover\ NdfR_{LMPt}} = \frac{\mathrm{EAF\ ^{15}N_{\ soil\ total\ N}(t)}}{\mathrm{EAF\ ^{15}N_{\ clover\ root\ N}(t)}} \quad (6)$$

The proportion of red clover NdfR was determined separately for each soil N pool (cf. Eq. 2) of the topsoil (0–0.25 m) and the subsoil (0.25–0.60 m).

The amount of red clover NdfR [g m^{-2}] accumulated in the respective microplot LMPt and layer was quantified according to

$$\mathrm{Clover\ NdfR_{LMPt}}\left[\mathrm{g\ m^{-2}}\right] = \mathrm{Soil\ total\ N_{LMPt}}\left[\mathrm{g\ m^{-2}}\right] \times \mathrm{proportion\ of\ clover\ NdfR_{LMPt}}. \quad (7)$$

Since LMP19 was only labelled during the second year, the entire amount of red clover NdfR accumulated during the two consecutive years (19 months) was calculated according to

$$\text{Clover NdfR(19)}\ \left[\text{g m}^{-2}\right] = \text{NdfR}_{\text{LMP8}}\ \left[\text{g m}^{-2}\right] + \text{NdfR}_{\text{LMP19}}\ \left[\text{g m}^{-2}\right]. \quad (8)$$

Red clover BGN [g m^{-2}] at the time t [months] of LMPt excavation was calculated according to

$$\text{Clover BGN(t)}\ \left[\text{g m}^{-2}\right] = \text{root N(t)}\ \left[\text{g m}^{-2}\right] + \text{NdfR(t)}\ \left[\text{g m}^{-2}\right] \quad (9)$$

where root N (t) represents physically recoverable root N at the time t and NdfR represents the accumulated amount of NdfR until the time t.

The proportion of red clover root N of non-classifiable roots at time t was calculated using the ^{15}N isotopic composition of the roots following the principles underlying the equation of Janzen and Bruinsma (1989):

$$\text{Proportion of clover root N(t)} = \frac{\text{EAF}\ ^{15}\text{N}_{\text{non-classifiable roots}}(t)}{\text{EAF}\ ^{15}\text{N}_{\text{clover root}}(t)}. \quad (10)$$

Red clover total N [g m^{-2}] accumulated until the time t was calculated by summing up AGN(t) and BGN(t):

$$\text{Clover total N(t)}\ \left[\text{g m}^{-2}\right] = \text{AGN(t)}\ \left[\text{g m}^{-2}\right] + \text{BGN(t)}\ \left[\text{g m}^{-2}\right]. \quad (11)$$

Total recovered perennial ryegrass N [g m^{-2}] accumulated until the time t comprising AGN and root N at the time t (NdfR was not determined) was calculated according to

$$\text{Grass total N(t)}\ \left[\text{g m}^{-2}\right] = \text{AGN(t)}\left[\text{g m}^{-2}\right] + \text{root N(t)}\left[\text{g m}^{-2}\right]. \quad (12)$$

The recovery of labelled (excess) ^{15}N of the assimilated ^{15}N urea solution of LMPt at the time t was calculated according to

$$^{15}\text{N recovery} = \frac{\left(\text{EAF}\ ^{15}\text{N total N(t)} \times \text{total N(t)}[\text{g m}^{-2}]\right)_{\text{clover}} + \left(\text{EAF}\ ^{15}\text{N total N(t)} \times \text{total N(t)}[\text{g m}^{-2}]\right)_{\text{grass}} + \left(\text{EAF}\ ^{15}\text{N total N(t)} \times \text{total N(t)}[\text{g m}^{-2}]\right)_{soil}}{\text{EAF}\ ^{15}\text{N assimilated urea solution(t)}\ [\text{g m}^{-2}] \times \text{total N assimilated urea solution(t)}\ [\text{g m}^{-2}]}. \quad (13)$$

Experimental design and statistical analyses

The experimental design was a split-split-plot situated in the Latin square of the DOK experiment, with four field replicates per treatment (Fließbach et al. 2007).

The data were fitted to a two factorial mixed effect model (treatment x time + error [paired plots, plot]) with an α level set at 0.05, below which the null hypothesis was rejected. Since treatments NOFERT and CONMIN2 on the one hand and BIOORG1 and BIOORG2 on the other hand were always situated adjacently in the Latin square design of the DOK experiment (Fließbach et al. 2007), *paired plots* were defined as a random factor. If the data per plot were obtained from more than one point of time or more than one microplot, *plot* was additionally used as second random intercept.

Prior to the statistical analysis, normal distribution of model residuals was tested using the Shapiro-Wilk test and, if violated, data were transformed, generally by log transformation. Compositional data were always centred log-ratio transformed according to Aitchison (1982) and Van den Boogaart and Tolosana-Delgado (2013). To detect differences between least significant means of the factors, a Student's t-test was applied as a posthoc test. Since clover had been almost eliminated by deer after the 2nd harvest (4 months), microplots located in the fourth replicate of treatment CONMIN2 had to be excluded.

Back-transformation of the centred log-ratio transformed data was calculated by CoDaPack version 2.01.15 (Thió-Henestrosa et al. 2009). Statistical analyses were carried out using the software package JMP® Pro10 (SAS Institute Inc., USA).

Results

Dry matter yields

At 19 months of cultivation, the dry matter yields of clover cumulative harvests ranged from 1570 g m^{-2} in NOFERT to 3820 g m^{-2} in BIOORG2. Clover yield differed significantly in the order NOFERT < BIOORG1 ≤ CONMIN2 ≤ BIOORG2. Cumulative grass yields ranged from 224 g m^{-2} in NOFERT to 890 g m^{-2} in CONMIN2. The contribution of stubbles to the overall dry matter production was small and constituted about 50 g m^{-2} for clover and 80 g m^{-2} for grass (Table 2). The clover proportion of the sward was high in all treatments, between 78% (CONMIN2) and 87% (NOFERT) of total dry matter yields.

Clover root dry matter increased with nutrient availability, from 186 g m^{-2} in NOFERT to 493 g m^{-2} in BIOORG2, similarly to the aboveground biomass whereas grass root dry matter, with an average of 145 g m^{-2}, did not show a clear response to the nutrient availability (Table 2).

Mineral nutrition of red clover and perennial ryegrass

Potassium concentration of clover increased from NOFERT to BIOORG1 to CONMIN2 and BIOORG2 (Table 3). Concentrations were highest in the weighted mean of the 1st to 2nd harvest (0 to 4 months) and decreased afterwards until the end of the experiment (3rd to 4th and 5th to 9th harvest), except for NOFERT. The K concentration of NOFERT remained constantly below the critical K concentration of 10 mg g^{-1} (Tucker and Smith 1952) throughout the two years, between 9.1 and 9.6 mg g^{-1}.

The N concentration of clover was not affected by treatments (Table 3) and was about 29 mg g^{-1}. Clover P concentrations were higher in CONMIN2 than in the two organic treatments BIOORG1 and BIOORG2 (Table 3). Generally, clover P concentrations were lower in the second year (5th to 9th harvest) compared to the first year (1st to 2nd and 3rd to 4th harvest). The limit of 2.2 mg g^{-1} (Davis 1991) was generally achieved, except for the cumulative 5th to 9th harvest in BIOORG2 (2.0 mg g^{-1}) and BIOORG1 (2.1 mg g^{-1}) (Table 3).

Nitrogen concentrations of grass were usually higher in CONMIN2 compared with the other treatments throughout the two years (Table 3). The critical N concentration of 25 mg g^{-1} for perennial ryegrass (Bolton et al. 1976) was only achieved with CONMIN2 at the weighted mean of the 5th to 9th harvest (Table 3).

The P concentration of grass was significantly different for the weighted mean of the 3rd to 4th harvest between NOFERT (3.7 mg g^{-1}) and BIOORG2 (4.9 mg g^{-1}) and the 5th to 9th harvest between CONMIN2 (4.5 mg g^{-1}) and the other treatments (2.7–3.2 mg g^{-1}) (Table 3). However, all treatments achieved the limit of the ryegrass critical P concentration of 2.5 mg g^{-1} (Bailey et al. 1997).

Table 2 Dry matter of cumulative 1st to 9th harvest of red clover and perennial ryegrass aboveground biomass grown in microplots (LMP19) for two consecutive years (0 to 19 months of sward cultivation) and the corresponding clover proportion and dry matter of stubble and roots at 19 months of sward cultivation

Treatment	Clover dry matter [g m^{-2}]			Grass dry matter [g m^{-2}]			Clover proportion [4]
	Cum. harvests [1]	Stubble [2, 3]	Root [2, 3]	Cum. harvests [1]	Stubble [2, 3]	Root [2, 3]	
NOFERT	1570 [c]	29	186 [c]	224 [b]	50	134	87%
BIOORG1	2900 [b]	33	271 [bc]	491 [a]	80	157	85%
BIOORG2	3820 [a]	61	493 [a]	709 [a]	111	114	84%
CONMIN2	3171 [ab]	68	467 [ab]	890 [a]	89	179	78%
SEM	29 ***	19 n.s.	47 *	85 *	12 n.s.	21 n.s.	2% n.s.

Mean of 4 (CONMIN2: n = 3); n.s. not significant, *, **, and *** significant at $p < 0.05$, < 0.01, and <0.001, respectively; same letters indicate no significant difference between treatments

[1] Cumulative dry matter of the 1st to 9th harvest during 2011 and 2012

[2] Dry matter at 19 months

[3] Data were log transformed

[4] Data were centred log-ratio transformed

Table 3 Nitrogen, phosphorus, and potassium concentrations as weighted mean of the 1st to 2nd, 3rd to 4th, and 5th to 9th harvest of red clover and perennial ryegrass

Harvests	Treatment	Clover nutrient concentration [mg g^{-1}] [1]			Grass nutrient concentration [mg g^{-1}] [1]		
		N [2]	P [3]	K [3]	N [2]	P [3]	K [3]
1st to 2nd (0 to 4 months)	NOFERT	27.9	2.5	9.1 ^{h}	19.6	3.4 ^{cde}	31.9 ^{de}
	BIOORG1	28.2	2.2	20.1 ^{ef}	20.1	3.5 ^{bcd}	48.3 ^{ab}
	BIOORG2	29.9	2.3	29.5 ^{a}	21.7	4.0 ^{abcd}	51.7 ^{a}
	CONMIN2	28.6	2.6	26.7 ^{ab}	24.0	3.6 ^{bcde}	50.7 ^{ab}
3rd to 4th (4 to 8 months)	NOFERT	29.5	2.4	9.6 ^{h}	20.5	3.7 ^{bcd}	27.8 ^{e}
	BIOORG1	27.6	2.3	16.5 ^{g}	24.6	4.3 ^{abc}	44.3 ^{b}
	BIOORG2	28.7	2.3	23.2 ^{bcd}	21.5	4.9 ^{a}	46.2 ^{ab}
	CONMIN2	29.6	2.4	21.3 ^{de}	24.0	4.0 ^{abcd}	42.8 ^{bc}
5th to 9th (8 to 19 months)	NOFERT	28.5	2.2	9.3 ^{h}	23.5	3.2 ^{de}	16.3 ^{f}
	BIOORG1	28.5	2.1	18.4 ^{fg}	23.6	2.7 ^{e}	30.2 ^{e}
	BIOORG2	27.8	2.0	22.5 ^{cde}	24.6	3.2 ^{de}	35.4 ^{d}
	CONMIN2	28.8	2.5	24.7 ^{bc}	25.7	4.5 ^{ab}	36.9 ^{cd}
	SEM p [4]	0.2 $^{n.s.}$	0.03 *	1.0 ****	0.4 **	0.1 **	1.6 ****
Treatment (T)		n.s.	*	****	*	n.s.	****
			NOFERT^{ab}	NOFERT^{c}	NOFERT^{b}		
			BIOORG1^{b}	BIOORG1^{b}	BIOORG1^{b}		
			BIOORG2^{b}	BIOORG2^{a}	BIOORG2^{b}		
			CONMIN2^{a}	CONMIN2^{a}	CONMIN2^{a}		
Harvests		n.s.	*	***	**	**	****
			1 + 2^{a}		1 + 2^{b}		
			3 + 4^{a}		3 + 4^{b}		
			5-9^{b}		5-9^{a}		
T x harvests		n.s.	n.s.	**	n.s.	*	*
Critical concentration		–	2.2-2.8 [5]	10 [6]	25 [7]	2.1 [8, 9]-2.5 [10]	18 [10]-28 [8]

Mean of $n = 4$ (CONMIN2: $n = 3$); n.s. not significant, *, **, ***, and **** significant at $p < 0.05$, < 0.01, <0.001, and <0.0001, respectively; same letters indicate no significant difference between factors

[1] Data were centred log-ratio transformed

[2] 1st to 2nd harvest from microplot LMP4; 3rd to 4th harvest from microplot LMP8; 5th to 9th harvest from microplot LMP19 (cf. Fig. 1)

[3] All harvests were taken from one microplot

[4] *p* value of the complete statistical model

[5] Davis (1991)

[6] Tucker and Smith (1952)

[7] Bolton et al. (1976)

[8] Smith et al. (1985)

[9] Liebisch et al. (2013)

[10] Dampney (1992)

Potassium concentrations of grass were generally lower in NOFERT compared with the other treatments and were also significantly lower in BIOORG1 than in BIOORG2 and CONMIN2 for the weighted mean of the 5th to 9th harvest (Table 3). Generally, the critical K concentration of 28 mg g^{-1} (Smith et al. 1985) was achieved in perennial ryegrass. Only treatment NOFERT for the weighted mean of the 5th to 9th harvest did not achieve the above-mentioned threshold (second cultivation period, 16.3 mg g^{-1}).

Enrichment and recovery of ^{15}N

The ^{15}N enrichment of clover parts ranged from about 2200 ppm to more than 9200 ppm EAF ^{15}N (Table 4, details Table S1). The ^{15}N enrichment of grass parts ranged from about 380 ppm to more than 4400 ppm EAF ^{15}N and that of soil from 12 ppm to 240 ppm EAF ^{15}N in the topsoil (0–0.25 m) and from 4 ppm to 26 ppm EAF ^{15}N in the subsoil (0.25–0.6 m). Generally, the ^{15}N enrichment was highest in clover shoots followed by clover roots, grass roots, grass shoots, the topsoil, and the subsoil. The recovery of applied ^{15}N ranged from about 60% to 80% at 4 and 8 months. At 19 months, when the plant-soil system was only labelled during the second year (Fig. 1, LMP19), the recovery of applied ^{15}N was lower, between 48% and 52%.

Above- and belowground nitrogen accumulation during sward cultivation

Clover AGN and BGN were clearly affected by the nutrient availability gradient increasing in the order NOFERT < BIOORG1 ≤ BIOORG2, except for CONMIN2 (Table S2, Fig. 2). Despite the highest N and P input to CONMIN2 (Table 1), amounts of AGN as well as BGN were similar to amounts of BIOORG1 (Table S2, Fig. 2). In contrast, grass AGN and root N were highest with sole mineral fertilisation in CONMIN2 but were comparable between half and full fertilised organic treatments, resulting in a treatment order NOFERT < BIOORG1 = BIOORG2 < CONMIN2 (Fig. 2, Table S2).

While the amounts of clover AGN increased over time (Fig. 2, Table S2), clover root N was highest at 8 months (Fig. 2, Table S2). At 19 months, only 70% of clover root

Table 4 Excess atom fraction (EAF) ^{15}N of red clover, perennial ryegrass, the topsoil (0–0.25 m), and the subsoil (0.25–0.6 m) as well as the recovery of applied ^{15}N at 4, 8, and 19 months of sward cultivation

Time (microplot)	Treatment	Clover EAF ^{15}N [ppm]		Grass EAF ^{15}N [ppm]		Soil EAF ^{15}N [ppm]		Recovery of applied ^{15}N [2)]
		Shoot [1)]	Root [2)]	Shoot [1)]	Root [2)]	0–0.25 m	0.25–0.6 m	
4 months (LMP4) [3)]	NOFERT	9017	3146	377	921	12	n.a.[3)]	61%
	BIOORG1	7029	2359	483	640	12	n.a.[3)]	82%
	BIOORG2	5507	2231	610	1183	15	n.a.[3)]	74%
	CONMIN2	8185	2731	530	875	19	n.a.[3)]	77%
	SEM	634	291	55	102	2	–	3%
8 months (LMP8)	NOFERT	9225	6756	3138	4469	64	26	73%
	BIOORG1	6999	7043	1921	2421	56	20	63%
	BIOORG2	8146	5624	1736	3178	67	18	83%
	CONMIN2	7999	5694	1295	2946	61	23	67%
	SEM	433	548	271	304	4	2	3%
19 months (LMP19)[4)]	NOFERT	5162	5455	1277	2108	121	13	52%
	BIOORG1	4901	3829	1476	2501	205	13	50%
	BIOORG2	3911	2895	1742	1949	240	4	51%
	CONMIN2	3696	3161	930	1505	143	8	48%
	SEM	252	386	107	257	15	2	2%

Mean of $n = 4$ (CONMIN2: $n = 3$); LMP = labelled microplot; n.a. not available

[1)] Weighted mean of cumulative harvests until time t (1st to 2nd harvest at LMP4, 1st to 4th harvest at LMP8, and 1st to 9th harvest at LMP19) and stubble at time t [2)]: EAF ^{15}N shoot = $\frac{\text{EAF } ^{15}\text{N stubble}_t \times \text{stubble}_t \text{ N} + \text{EAF } ^{15}\text{N} \sum_{i=1}^{n} \text{harvest}_i \times \text{N} \sum_{i=1}^{n} \text{harvest}_i}{\text{stubble}_t \text{ N} + \text{N} \sum_{i=1}^{n} \text{harvest}_i}$

[2)] Mean at the time of LMP excavation

[3)] No subsoil sampling at 4 months

[4)] 1st to 4th harvests remained unlabelled. Details for single harvest ^{15}N enrichments see Table S1

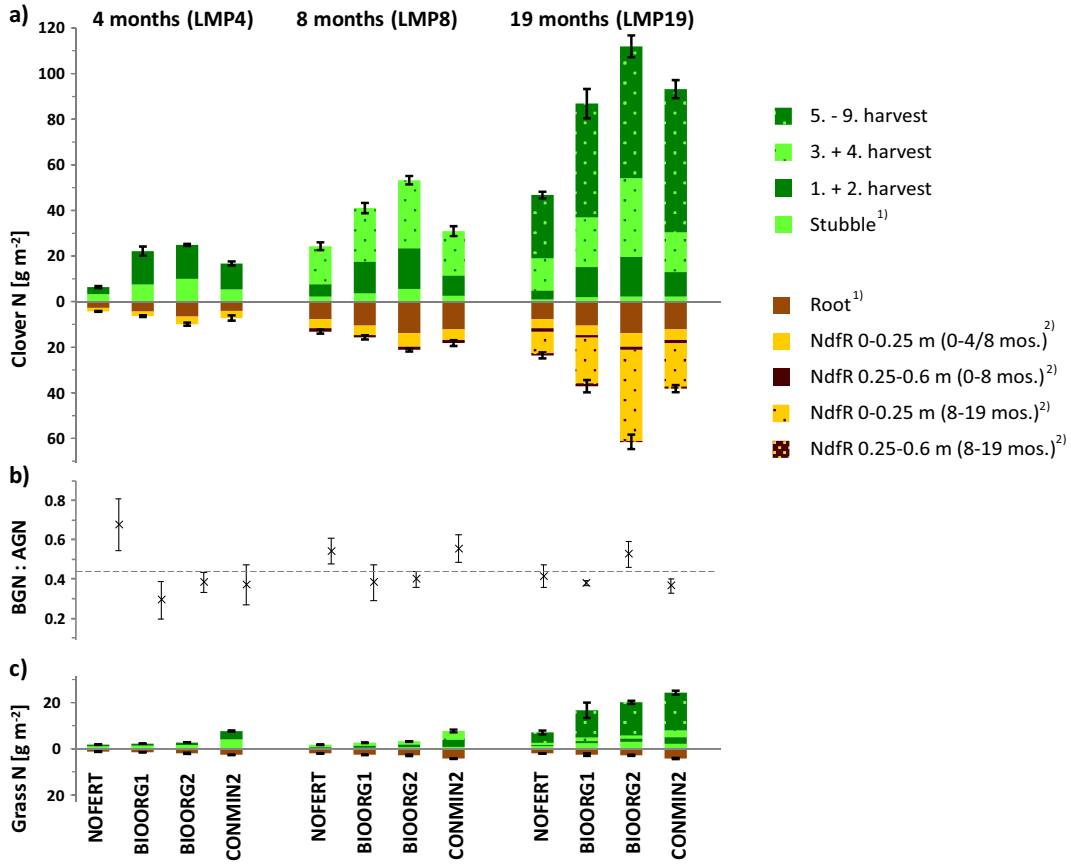

Fig. 2 Allocation of red clover belowground N (BGN) and aboveground N (AGN) (**a**), red clover BGN to AGN ratio (**b**), and allocation of perennial ryegrass root N and AGN (**c**) at 4 months (1st to 2nd harvest, stubble[1)], root[1)], and N derived from rhizodeposition[2)] [NdfR] in 0–0.25 m), 8 months (1st to 4th harvest, stubble[1)], root[1)], and NdfR[2)] in 0–0.6 m [end of the first year]), and 19 months of sward cultivation (1st to 9th harvest, stubble[1)], root[1)], and NdfR[3)] in 0–0.6 m [end of the second year]) as mean ($n = 4$ [CONMIN2: $n = 3$]) +/− *SEM*. Columns above and below the x-axis represent AGN and BGN / root N, resp. (2a and 2c); clover NdfR was not determined in 0.25-0.6 m at 4 months of cultivation; no roots were detected in the subsoil (0.25–0.6 m); grass NdfR was not determined. [1)] Standing biomass N at the time of excavation [2)] At the time of microplot excavation [3)] Sum of NdfR at the times 8 and 19 months (cf. Eq. 8)

N found at 8 months was recovered. In contrast, clover NdfR increased three- to more than six-fold between 8 and 19 months. In consequence, clover BGN increased on average by a factor of 2 (Fig. 2, Table S2).

Clover accumulated BGN between 4 and 10 g m^{-2} at 4 months, 13 and 21 g m^{-2} at 8 months, and 19 g m^{-2} (NOFERT), 32 g m^{-2} (BIOORG1), 35 g m^{-2} (CONMIN2), and 58 g m^{-2} (BIOORG2) at 19 months (Fig. 2, Table S2). The amount of clover NdfR increased from 1.5 to 3.1 g m^{-2} at 4 months to 5 to 7 g m^{-2} at 8 months to 16 g m^{-2} (NOFERT), 25 to 26 m^{-2} (BIOORG1, CONMIN2), and 47 g m^{-2} (BIOORG2) at 19 months (Fig. 2, Table S2).

While amounts of grass AGN increased over time (Fig. 2, Table S2) as clover AGN did, grass root N peaked at 8 months, as observed for clover root N (Fig. 2, Table S2). At 19 months, only about 60% of grass root N found at 8 months was recovered.

The percentage of clover N of total sward N was high (Fig. 2, Table S2), between 69% (CONMIN2 at 4 months) and 93% (BIOORG2 at 8 months). However, the clover N percentage in CONMIN2 was 5% to 19% lower compared to the other treatments (69% at 4 months, 81% at 8 months, and 83% at 19 months).

Allocation of red clover nitrogen and nutrient availability

The distribution between clover AGN and BGN was never significantly affected by the treatments during the

entire study (Table S2). Clover BGN constituted about 40% of AGN at all points of time (Fig. 2, Table S2).

The size of clover BGN fractions strongly changed over time both in terms of absolute amounts and of relative proportions (Table 5). While clover macro root N (> 3 mm) decreased from 8 to 19 months, clover rootlets N (< 3 mm) and NdfR increased in the meantime (Table 5). At 4 and 8 months, the contribution of clover NdfR to BGN was relatively low, between 30% and 40%. However, at 19 months, clover NdfR was the major part of clover BGN, ranging from 72% to 82% of clover BGN (Fig. 2, Table S2). In parallel, the proportion of clover rootlets N (< 3 mm) of clover total root N was about two times greater at 19 months compared with 8 months (Table 5).

No roots could be found in the subsoil (0.25–0.6 m). However, clover NdfR was found in the subsoil, ranging from 1.0 to 1.4 g m^{-2} at 8 months and 1.4 to 2.2 g m^{-2} at 19 months. At 8 months, a comparatively large proportion of about 30% of total clover NdfR was recovered in the subsoil, whereas at 19 months the proportion constituted only about 10% (Table 5).

Table 5 Allocation of red clover belowground N to roots and rhizodeposition (NdfR) in the topsoil (0–0.25 m) and subsoil (0.25–0.6 m) at 4, 8, and 19 months of sward cultivation

Time	Treatment	Clover root N [g m^{-2}]		Clover NdfR [g m^{-2}]	
		>3 mm [1, 2)]	< 3 mm and > 0.5 mm [1)]	0–0.25 m [3)]	0.25–0.6 m [2, 4)]
4 months	NOFERT	1.4	1.3 [e]	1.5 [g]	n.a.
	BIOORG1	2.6	1.8 [de]	1.8 [f]	n.a.
	BIOORG2	3.9	2.3 [cde]	3.1 [efg]	n.a.
	CONMIN2	2.3	1.8 [cde]	2.5 [efg]	n.a.
8 months	NOFERT	4.7	2.5 [bcd]	3.9 [def]	1.4
	BIOORG1	8.2	2.0 [cde]	3.8 [deg]	1.0
	BIOORG2	11.3	2.4 [cde]	6.0 [d]	1.0
	CONMIN2	10.2	2.3 [cde]	4.1 [defgh]	1.3
19 months	NOFERT	1.9	1.7 [de]	13.3 [c]	2.2
	BIOORG1	2.4	2.9 [bc]	24.2 [b]	2.0
	BIOORG2	5.1	5.4 [a]	46.2 [a]	1.4
	CONMIN2	6.7	3.6 [b]	22.7 [b]	2.1
	SEM p [5)]	0.5 ***	0.2 ****	2.1 ****	0.2 *
Treatment (T)		*	*	**	n.s.
		NOFERT [b]			
		BIOORG1 [ab]			
		BIOORG2 [a]			
		CONMIN2 [a]			
Period		****	****	****	***
		4 months [c]			–
		8 months [a]			8 [b]
		19 months [b]			19 [a]
T x Period		n.s.	**	****	n.s.

Mean of $n = 4$ (CONMIN2: $n = 3$); n.s. not significant, *, **, ***, and **** significant at $p < 0.05$, < 0.01, < 0.001, and <0.0001, respectively; n.a. not available; same letters indicate no significant difference between factors

[1)] No roots were discovered in the subsoil

[2)] Data were log transformed

[3)] Data were square root transformed

[4)] No subsoil sample was taken at 4 months of cultivation

[5)] *p* value of the complete statistical model

Discussion

Evolution of red clover N partitioning between AGN and BGN over time

Clover BGN was highest at the end of the second year, at 19 months (Fig. 2). While NdfR increased from one excavation time to the following one, root N was greatest at the end of the first year (8 months). The significantly lower amount of root N at the end of the second compared with the end of the first year may be due to enhanced root decay and reduced root N regrowth (Chen et al. 2016). Root decay is indicated by a lower ratio of root N > 3 mm to rootlets N < 3 mm at the end of the second compared to the end of the first year (Table 5). Bowley et al. (1984) stated that red clover taproots normally disintegrate during the second year, which is supported by our results. In parallel, the amount of NdfR increased by a factor of 3 (NOFERT) to more than 6 (BIOORG2) between the end of the first and the end of the second year (Fig. 2). Decreasing root N together with increasing NdfR suggests that root N turnover was higher than regrowth of new roots during the second year. As a result, BGN development was reflected neither by the development of root N nor by the development of NdfR alone. Hence, both root N and NdfR are essential to obtain realistic BGN estimates.

Only a few studies have focused on red clover BGN in clover-ryegrass swards. In a red clover-perennial ryegrass sward cultivated during one growing season, Dahlin and Stenberg (2010b) found barely 60% of the amount of clover AGN and about 20% of the amount of clover root N, but three times the amount of clover NdfR compared to results of treatment CONMIN2 in the present study at 8 months of cultivation. However, the amount of clover BGN determined in our study in the respective treatment (Table S2) is comparable to the amount determined by Dahlin and Stenberg (2010b). The difference in amounts of clover NdfR and root-N may partly be related to different methods used for root separation, which may have affected the relation between clover root N and NdfR. While Dahlin and Stenberg (2010b) separated roots and soil by using a 2 mm sieve, we implemented a 0.5 mm sieving subsequent to the 3 mm sieving and recovered an average amount of clover rootlets < 3 mm of 2.4 g m^{-2}. Fine roots recovered with this procedure additionally contributed 18% of clover root N and reduced clover NdfR by 26% (Table 5) compared to amounts recovered by 3 mm sieving alone. However, the sieving protocol did neither change the BGN to AGN ratio nor total N, but the proportion of root N and NdfR. Furthermore, differences in site factors, varieties, and fertilisation (e.g. 160% of N but 60% of P and 20% of K applied with fertilisation by Dahlin and Stenberg 2010b) might have led to differences in sward performance and belowground inputs.

Nutrient availability did not affect the BGN to AGN ratio of red clover

To evaluate nutrients that limit plant growth primarily, Römheld (2012) suggested critical nutrient concentrations in plants and McNaught and During (1970) nutrient norm ratios. We focused on N, P, and K concentrations, since screenings in a previous study (Oberson et al. 2013) revealed no limitations of other nutrients (Oberson, unpublished results).

While the P and K concentrations of cumulative clover harvests were signficantly affected by treatments, only the K concentration clearly reflected the treatment related K availability of the soil (Table 1 and Table 3). However, low K availability only seems to have limited clover growth in NOFERT, as suggested by low K concentrations (Tucker and Smith 1952) throughout the two consecutive cultivation years. Phosphors, by contrast, only limited clover growth in the both organic treatments BIOORG1 and BIOORG2 during the second cultivation year as indicated by the P concentrations (Davis 1991) together with the N to P ratios (Fystro et al. 2008) of the cumulative 5th to 9th harvests.

The N and K concentrations of cumulative grass harvests were signficantly affected by treatments. However, only K limited grass growth in NOFERT during the second cultivation year (2012) as suggested by the low K concentration, the low K to P ratio (Liebisch et al. 2013), and the high N to K ratio (Dampney 1992) of the cumulative 5th to 9th harvest. We assume that grass was generally sufficiently supplied by P and N except for N in BIOORG2 in 2011 as suggested by low N concentrations (Bolton et al. 1976) together with low N to P ratios (Liebisch et al. 2013) and low N to K ratios (Dampney 1992) of cumulative harvests in 2011.

In a previous study, Oberson et al. (2013) found similar nutrient limitations in clover-grass swards growing in DOK field plots. Both data sets clearly revealed that red clover and to a lesser degree grass were primarily K limited in NOFERT.

With rising nutrient availability, we expected a relative increase of clover AGN compared to clover BGN, corresponding to a decrease in the BGN to AGN ratio. However, the ratio was similar for all treatments at 4, 8, and 19 months of cultivation (Fig. 2, Table S2), with an average around 0.4 irrespective of the treatment, i.e. the nutrient availability. Hence, red clover BGN could roughly be estimated from AGN under field conditions independently of the management intensity and the cultivation time, using a factor of 0.4. The factor of 0.4 is below the range of factors reviewed by Herridge et al. (2008) for fodder legumes, ranging from 0.5 to 2.1. The authors proposed an average factor of 1.0 for fodder legumes to calculate BGN from AGN, but the data set did not include red clover. However, Dahlin and Stenberg (2010b) found a similar factor of 1.1 for red clover in a red clover-perennial ryegrass mixture.

We have not included the N-transfer from clover to grass, which removes clover NdfR from the soil. Thus, N-transfer to associated grass will contribute to gross clover BGN and will potentially increase the factor of 0.4. For instance, in a two-year-old clover-grass mixture growing in the DOK experiment, about 50% of grass N derived from clover (Oberson et al. 2013).

Dry matter yields in microplots versus DOK field plots

Dry matter yields of our simple two-component red clover-perennial ryegrass model sward were higher compared with the clover-grass mixture of the field plots (Table 2, Table S3). Field plots yielded between 1270 and 2400 g m^{-2} and the clover proportion on a dry matter basis was between 30% and 52%. These yields and clover proportions were comparable to those of previous years (Oberson et al. 2013). Dry matter yields in microplots were about 1.5 to 2.1 fold higher than in field plots, however, while clover yielded up to 4.5 times as much, grass yielded only about half. We attributed the higher dry matter yields of clover to the substitution of the red clover varieties 'Merian' and 'Global' with the more productive, competitive, and persistent variety 'Dafila' (Suter et al. 2014) and to the simplification of the sward because white clover has lower yields than red clover (Oberson et al. 2013). Lower grass dry matter yields in the microplots were thus the result of the very strong clover competition. In consequence, dry matter yields of the sward in the microplots were highest in BIOORG2, whereas in the field plot the higher yields were found in CONMIN2. Finally, higher yields in microplots than surrounding field plots have been reported previously, e.g. by Oberson et al. (2007) for soybean, suggesting that additional factors such as protection by the microplot frame, border effects, careful management of small experimental units, and scale may affect the yields. Therefore, upscaling of our data should take into account the clover proportion and N yield measured at more realistic field scales.

Methodological considerations

To detect red clover NdfR in the bulk soil, a sufficient ^{15}N enrichment of the soil via rhizodeposition from ^{15}N labelled roots is required (Wichern et al. 2008). With a target enrichment of 5000 ppm EAF ^{15}N of clover roots, we achieved a sufficient bulk soil enrichment, between 12 (LMP4) and 240 ppm (LMP19) EAF ^{15}N in the topsoil (0–0.25 m) and between 4 ppm and 26 ppm EAF ^{15}N in the subsoil (0.25–0.6 m) (Table 4). Grass roots and shoots showed high ^{15}N enrichments, ranging from 377 ppm (shoot NOFERT, LMP4) to 4469 ppm EAF ^{15}N (root NOFERT, LMP8), thus indicating a strong BGN transfer from clover to grass (Oberson et al. 2013) due to a fast turnover of red clover NdfR (Hamilton et al. 2008; Haystead and Marriott 1979).

The recovery of labelled ^{15}N was generally in the medium range compared to similar studies with red clover-perennial ryegrass swards under field conditions. While Rasmussen et al. (2007) recovered only 25% of applied ^{15}N after 65 days of labelling, Høgh-Jensen and Schjoerring (2001) could recover 85% of applied ^{15}N after two consecutive growing seasons. However, we removed the highly ^{15}N labelled leaves with their stalks after completion of tracer uptake, but did not account for the removed ^{15}N amount contained therein when calculating the recovery (Eq. 13). In addition, gaseous ammonia losses from the leaves into the atmosphere (Gooding and Davies 1992) and losses from red clover NdfR by denitrification or nitrate leaching into deeper soil layers below 0.6 m (Robertson and Vitousek 2009) may explain ^{15}N label losses. Higher precipitation during the second compared with the first year (Fig. S2) might have resulted in greater leaching of red clover NdfR into deeper soil layers and higher denitrification rates, due to oxygen limiting conditions as a consequence of increased soil moisture (Butterbach-Bahl et al. 2013).

The principle underlying Eq. 6 for calculating the proportion of clover NdfR assumes that i) clover NdfR has the same ^{15}N enrichment as clover root N (Janzen

and Bruinsma 1989). This implies that the ^{15}N enrichment of the root is constant over ii) space (Jensen 1996) and iii) time (Sawatsky and Soper 1991). Leakage of highly ^{15}N enriched tracer or tracer metabolites shortly after labelling is one of the main factors violating assumption i), leading to an overestimation of NdfR (Gardner et al. 2014; Gasser et al. 2015). Gasser et al. (2015) found 0.5% of applied ^{15}N enriched tracer or metabolites leaked after one day from mono-cropped red clover in a model greenhouse study, leading to a vast overestimation of NdfR. However, the bias would decrease if clover or the competitive grass partner (Snaydon and Howe 1986) reabsorbed soluble tracer ^{15}N, which is suggested in our case by the fast N transfer from clover to grass after the first labelling in samples taken at 4 months of cultivation (Table 4, Table S1). Thus, we assume that biases from possible tracer leakage in soil were small. Gasser et al. (2015) found spatial root enrichments varying by a factor of 3, hence violating assumption ii). The observed non-uniform ^{15}N labelling of the root (e.g. Gasser et al. 2015; Khan et al. 2002; Russell and Fillery 1996) might be partially the result of symbiotic N_2 fixation diluting the ^{15}N enrichment of nodulated compared to unnodulated root segments (Russell and Fillery 1996). However, since defoliation of red clover also results in nodule turnover (Bowley et al. 1984), the average ^{15}N enrichment of the nodulated root should be comparable with the average ^{15}N enrichment of NdfR. This assumption is supported by Gasser et al. (2015), who detected only small biases of NdfR due to the spatial heterogeneity of the root ^{15}N enrichment. Therefore, we sampled the entire root and homogenised the ground samples before the analyses to minimise the spatial bias in ^{15}N enrichment of the clover root. Assumption iii), equal root enrichment over time, was not met in the first year, as root ^{15}N enrichment recorded at 4 and at 8 months differed by a factor of 2, although we adjusted the multiple pulse labelling to the predicted N uptake. Gasser et al. (2015) and Sawatsky and Soper (1991) concluded that biases of NdfR estimations should be small if the ^{15}N enrichment of the root at the time of sampling is comparable with the mean root enrichment over time. However, the nature of the clover grass sward is very complex, due to species mixtures, frequent harvests inducing root turnover, and tracer removal by harvests. Induced root turnover releases soluble ^{15}N enriched compounds to the rhizosphere, which can be taken up by either grass or, less likely, due to the strong competition of grass (Nyfeler et al. 2011), by clover immediately or subsequently to the mineralisation (Gasser et al. 2015; Hart et al. 1986; Mayer et al. 2003).

Because we did not know how these processes affect clover NdfR estimations in a two year clover grass sward, we split the determination of NdfR on two microplots, one being labelled in the first and one being labelled in the second year (Fig. 1). Both results were then summed to determine clover NdfR over 19 months of cultivation (Eq. 8). As a control, we used a second microplot (LMP19B, Table S4), which was ^{15}N labelled during the first year according to LMP8, but was not further labelled during the second year. The LMP19B was excavated at the same time as LMP19. In LMP19B, the ^{15}N enrichment of the clover root probably decreased continuously during the second year, as suggested by the decreasing aboveground ^{15}N enrichment of the grass partner (data not shown). Clover root N decrease was the result of i) dilution due to regrowth of new root biomass with unlabelled N from symbiotic N_2 fixation or from uptake of soil N and ii) of tracer ^{15}N removal from the system by harvesting aboveground biomass. Therefore, clover NdfR of LMP19B was calculated using the geometric mean of clover root N EAF ^{15}N at 8 (LMP8) and at 19 (LMP19B) months. Clover NdfR differed only in treatment BIOORG2 between the labelling strategies, with 48 g m^{-2} in LMP19 and 21 g m^{-2} in LMP19B (Table S4). This might partly be the result of about 40% higher AGN accumulations in LMP19 (112 g m^{-2}) than in LMP19 B (82 g m^{-2}) in BIOORG2. However, the ratios BGN to AGN were not significantly different between treatments and labelling strategies.

Conclusions

During two consecutive years of red clover-perennial ryegrass sward cultivation, dry matter yields and total N uptake responded to varying nutrient availabilities established by organic and conventional cropping systems. Potassium availability was the main growth-limiting factor for red clover in treatments with low nutrient availability. In contrast, organic treatments were limited in P during the second cultivation year. However, independently of the nutrient availability, red clover BGN increased proportionally to cumulative AGN. Hence, red clover BGN could be estimated from red clover AGN by multiplying AGN with a factor of 0.4.

The increase of red clover BGN with time was the result of a strong increase of red clover NdfR between 8 and 19 months, the latter thus compensating the decrease of root N between these two time points. Consequently, red clover BGN was reflected neither by the development of root N nor by the development of NdfR. The inverse relation between red clover root N and NdfR development between 8 and 19 months was probably triggered by enhanced root turnover, resulting in a distinct absolute and relative increase of NdfR until the end of the second year, independently of the nutrient availability.

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