



Seasonal variation in nitrogen pools and $^{15}\text{N}/^{13}\text{C}$ natural abundances in different tissues of grassland plants

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Abstract. Seasonal changes in nitrogen (N) pools, carbon (C) content and natural abundance of ^{13}C and ^{15}N in different tissues of ryegrass plants were investigated in two intensively managed grassland fields in order to address their ammonia (NH_3) exchange potential. Green leaves generally had the largest total N concentration followed by stems and inflorescences. Senescent leaves had the lowest N concentration, indicating N re-allocation. The seasonal pattern of the Γ value, i.e. the ratio between NH_4^+ and H^+ concentrations, was similar for the various tissues of the ryegrass plants but the magnitude of Γ differed considerably among the different tissues. Green leaves and stems generally had substantially lower Γ values than senescent leaves and litter. Substantial peaks in Γ were observed during spring and summer in response to fertilization and grazing. These peaks were associated with high NH_4^+ rather than with low H^+ concentrations. Peaks in Γ also appeared during the winter, coinciding with increasing $\delta^{15}\text{N}$ values, indicating absorption of N derived from mineralization of soil organic matter. At the same time, $\delta^{13}\text{C}$ values were declining, suggesting reduced photosynthesis and capacity for N assimilation. $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values were more influenced by mean monthly temperature than by the accumulated monthly precipitation. In conclusion, ryegrass plants showed a clear seasonal pattern in N pools. Green leaves and stems of ryegrass plants generally seem to constitute a sink for NH_3 , while senescent leaves have a large potential for NH_3 emission. However, management events such as fertilisation and grazing may create a high NH_3 emission potential even in green plant parts. The obtained results provide input for future modelling of plant-atmosphere NH_3 exchange.

1 Introduction

Nitrogen (N) is a constituent of compounds such as amino acids, proteins, RNA, DNA and several phytohormones and is thereby an essential macroelement for plants. The supply of N has a profound influence on many aspects of plant growth and development, including the growth of roots and shoots (Hirel et al., 2007). Nitrogen is considered to be the nutrient which most widely limits the growth of vegetation in terrestrial ecosystems (Vitousek and Howarth, 1991; Xia and Wan, 2008). Due to nitrogen losses associated with anthropogenic activities, in particular synthesis and application of N fertilizers, animal production and fuel combustion, the amount of nitrogen entering the biosphere has increased dramatically since the industrial revolution in the 1860s (Frink et al., 1999; Erismann et al., 2008). Ammonia (NH_3) is an important component of this increase and is becoming recognized as a reactive N pollutant in the atmosphere with impacts on a series of ecological problems such as eutrophication, acidification, alteration of biodiversity and global warming (Sutton et al., 1998; Dragosits et al., 2002; Krupa, 2003; Allen et al., 2011).

On a global basis, NH_3 exchange between vegetated surface and atmosphere is an important process in the N cycle and also a key uncertainty in quantifying atmospheric NH_3 and N depositions to terrestrial ecosystems (Pilegaard et al., 2009). Ammonia emissions generally occur in intensively managed agricultural ecosystems, while semi-natural ecosystems are more likely to act as NH_3 sinks (Sutton et al., 1993, 1994; Schjoerring et al., 1998, 2000).

Grasslands are one of the major cropping ecosystems (Bussink et al., 1996). It has been shown that NH_3 fluxes

over grassland appear to be bi-directional. In non-fertilized agricultural grassland in The Netherlands, NH_3 emission episodes covered about 50% of the time during a warm and dry summer period of 28 days. In contrast, during a wet and cool autumn period of 31 d, NH_3 depositions were frequent, covering about 80% of the time (Kruit et al., 2007). In intensively managed grasslands, fertilizers represent the major source of N input, and repeated cuttings and/or continuous grazing are the major normal management practices. Large NH_3 emissions may be recorded after N fertilization (Harper et al., 1996; Herrmann et al., 2001; Milford et al., 2009; Højito et al., 2010), slurry application (Flechard et al., 2010) and also following cutting of the grass canopy (Milford et al., 2009). Grazing events may also have effects on grassland NH_3 fluxes (Loubet et al., 2002) and N status (van Hove et al., 2002; Li et al., 2010) and may promote availability of N in N-limited grasslands by increasing N cycling and NO_3^- assimilation (Frank and Evans, 1997). Within the canopy, different tissues of the grass plants contribute differently to NH_3 fluxes (Herrmann et al., 2009). The senescent leaves are recognized as the strongest source and green leaves can recapture NH_3 emitted in deeper layers of the canopy (David et al., 2009). Taken together, the direction and magnitude of NH_3 exchange over grasslands thus depend on management practices, climatic conditions and canopy growth, and all of these have a close relationship with N status and turnover in the plants (Schjoerring et al., 1998, 2000; Massad et al., 2008).

Although recent NH_3 exchange models have incorporated aspects of the N metabolism of plants (Massad et al., 2008; Personne et al., 2009), more information about the dynamics of plant N pools is essential to develop improved mechanistic models for better prediction of plant-atmosphere NH_3 exchange in terrestrial vegetation (Massad et al., 2010). Most of the field investigations which previously have reported seasonal variations in N status and turnover of annual (Herman et al., 2003) and perennial grasslands (Harper et al., 1996; Loubet et al., 2002; Mattsson et al., 2009) have been limited to the period of optimum growth, i.e. from mid-spring to early autumn. Few studies have spanned the entire season which is important because the processes involved in internal N turnover are closely linked to plant phenology (van Hove et al., 2002). For instance, the relative importance of N remobilization from roots and/or leaves versus absorption of soil N depends on the growth stage (Bausenwein et al., 2001; Santos et al., 2002; Gloser, 2005). It is therefore essential to investigate the N status and turnover at the annual scale to provide supplementary data to NH_3 exchange models.

Measurements of changes in the natural abundance of stable isotopes provide a strong tool for studies of plant N and C dynamics (Dawson et al., 2002; Aerts et al., 2009; Ballantyne et al., 2011; Brüggemann et al., 2011). The natural abundance of ^{13}C ($\delta^{13}\text{C}$) in plant leaves reflects net CO_2 assimilation and stomatal conductance which relate to CO_2 uptake and diffusion, respectively (Farquhar et al., 1982; Farquhar et

al., 1989; Brüggemann et al., 2011). Based on this, $\delta^{13}\text{C}$ can be extended to indicate plant water use efficiency (Werner et al., 2011) and environmental conditions such as precipitation (Fotelli et al., 2003), temperature (Salmon et al., 2011) and ground water availability (Máguas et al., 2011). The natural abundance of ^{15}N ($\delta^{15}\text{N}$) in plant leaves reflects the form of inorganic nitrogen absorbed as well as its origin (Robinson, 2001). Based on compilation of data for over 11000 plant species across the world, Craine et al. (2009b) proposed that foliar $\delta^{15}\text{N}$ is correlated with foliar N concentration, mean annual precipitation and temperature. Accordingly, studies of changes in the natural abundance of C and N isotopes can be used to reveal plant responses to climate change (Aerts et al., 2009).

Within the framework of the NitroEurope integrated project, intensive measurements of NH_3 fluxes and meteorological parameters have been undertaken at field sites including forest, grassland, arable land and wetland/shrubland ecosystems (Skiba et al., 2009). The objective of the present work was to study nitrogen pools, carbon content and natural abundance of ^{13}C and ^{15}N in different grass tissues in order to address their seasonal pattern in relation to the NH_3 exchange potential of different tissues of ryegrass. The obtained results will serve as input for future modelling of plant-atmosphere NH_3 exchange.

2 Materials and methods

2.1 Site description and sampling

The sites were two intensively managed grassland fields ("North" and "South" field according to their relative position) located at Easter Bush, Edinburgh, Scotland ($55^\circ 52' \text{N}$, $03^\circ 02' \text{W}$; 190 m above sea level). The soil type was clay loam and the dominating plant species was ryegrass (*Lolium perenne* >90%; Sutton et al., 2001; Loubet et al., 2002; Skiba et al., 2009). During the experimental period, i.e. from April 2008 to June 2009, the mean temperature and total precipitation were 8.9°C and 1009 mm, respectively (Fig. 1). Both fields received 225 kg N ha^{-1} in NH_4NO_3 and 196 kg N ha^{-1} in urea in total on the dates specified in Table 1. Sheep and lambs grazed at an average stocking density of 0.8 and 0.92 livestock units (LSU) ha^{-1} at the North and South field, respectively (Table 1). There was no long-term continuous grazing (more than one month) from January to mid-March and February to early-March 2009 for the North and South fields, respectively (Fig. 1). Ryegrass sampling was carried out in four replicate 1 m^2 sub-plots (two in North and two in South field) once a month and subsequently divided into green leaves, stems, inflorescences (if present), senescent leaves (senescing attached leaves) and litter (senescent or dead detached leaves). All plant samples were immediately frozen upon sampling and kept at -80°C

Table 1. Dates of sampling and agronomic management (N application and grazing) in North and South fields. Data provided by Centre for Ecology & Hydrology, Edinburgh, Scotland.

Date					
Sampling	Management	N application (kg N ha ⁻¹)	North field	South field	
16/04/08	19/11/08	04/04/08	NH ₄ NO ₃	69	69
14/05/08	17/12/08	13/05/08	NH ₄ NO ₃	52	52
16/06/08	12/01/09	18/06/08	NH ₄ NO ₃	52	52
16/07/08	20/02/09	29/07/08	NH ₄ NO ₃	52	52
12/08/08	13/03/09	28/08/09	Urea	35	35
17/09/08	21/04/09	17/03/09	Urea	92	92
21/10/08	11/05/09	12/05/09	Urea	69	69
	22/06/09				
Stocking density (LSU ha ⁻¹)			North field	South field	
	16/04/08–	Mean	0.80	0.92	
	22/06/09	Max	2.85	2.79	

until lyophilized. The samples collected in December 2008 were not analyzed due to contamination by soil.

2.2 Analysis of plant tissues

Details of the analytical procedures can be obtained from Husted et al. (2000a), Mattsson et al. (2009) and Wang et al. (2011). In brief, chlorophyll was extracted from frozen plant materials by the use of methanol. Lyophilized samples were used for analysis of total C and N and signatures of ^{13}C and ^{15}N natural abundances ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) by mass spectrometry in a system consisting of an ANCA-SL Elemental Analyzer coupled to a 20-20 Tracermass Mass spectrometer (SerCon Ltd., Crewe, UK). Plant tissues were ground in 10 mM formic acid for analysis of bulk tissue NH_4^+ and NO_3^- , followed by fluorometric detection of NH_4^+ after derivatization with o-phthalaldehyde and spectrophotometric detection of NO_3^- in a flow injection system (Lachat 8000 series, Hach, Loveland, Colorado). Formic acid tissue extracts were also analysed for total soluble N concentration using the same instrument as for measurement of total C and N. For determination of bulk tissue pH, plant tissues were homogenized in milli-Q water, centrifuged and pH measured in the supernatant by use of a microelectrode (Metrohm, Herisau, Switzerland).

2.3 Calculations and statistical analysis

Isotopic ratios of carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) were calculated using the following equation:

$$\delta^{13}\text{C} \text{ or } \delta^{15}\text{N} = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000$$

where R is the $^{13}\text{C}:^{12}\text{C}$ or $^{15}\text{N}:^{14}\text{N}$ ratio and the standard is Pee Dee Belemnite (PDB) for carbon and atmospheric air for nitrogen.

Data were analysed by one-way analysis of variance combined with Duncan's tests. Correlation analyses were based

on Pearson's product moment if the data met the normality requirements in the Shapiro-Wilk test; otherwise Spearman's rank order was used.

3 Results

3.1 Tissue biomass

The total fresh above-ground biomass was 121 g m⁻² at the first sampling in April 2008 (Fig. 2). It increased rapidly to the maximum of 628 g m⁻² after two months and remained at a relatively high level until November followed by a rapid decrease. During winter 2008 and spring 2009 (November–March), the biomass remained at a relatively low level until a rapid increase again in April 2009. The inflorescences started to appear in May of both years and lasted about six months in 2008. From April to June, the senescent leaves and litter accounted for no more than 10 % of the total fresh biomass in both years. In the autumn, the litter proportion increased to around 30 % and increased even further to >50 % during the winter (Fig. 2).

3.2 Tissue relative water content and leaf chlorophyll content

The relative water content of green leaves and stems was rather constant around 75 % throughout the season (Fig. 3a). Inflorescences contained around 67 % water, while the relative water content of senescent leaves and litter fluctuated between 30 and 80 %, reflecting loss of regulatory function.

Green leaves showed declining chlorophyll content and chlorophyll a/b ratio during spring and summer 2008, but thereafter remained constant during the winter (Fig. 3b). In spring 2009, the values increased again to a level similar to that in the spring-summer season of 2008 (Fig. 3b).

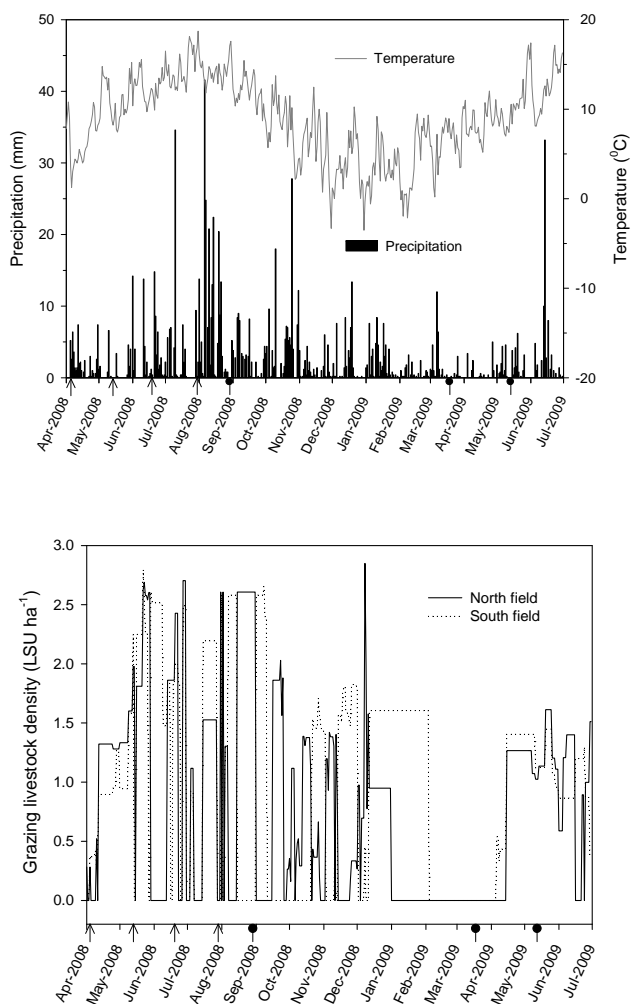


Fig. 1. Seasonal variation in temperature (grey line) and precipitation (black columns) (A), and grazing livestock density in north (solid line) and south field (dots) (B). Arrows below the X-axis represent N application as NH_4NO_3 ; the bar with solid circle represents N application as urea. Data provided by Centre for Ecology & Hydrology, Edinburgh, Scotland.

3.3 Tissue total nitrogen and carbon concentration

Green leaves had at all harvest occasions the highest N concentration compared with the other tissues (Fig. 4). The lowest N concentration was generally observed in senescent leaves and litter. The N concentration in all tissues gradually decreased from April to July 2008 and thereafter remained relatively constant over the rest of the season. Peak maximum N concentrations of 4.8 % and 2.9 % for green leaves and stems, respectively, were reached early 2009. The N concentration in spring 2009 was lower than that in spring 2008 (Fig. 4).

The C concentration in green leaves showed little seasonal variation, remaining around 39–42 % on a dry matter basis (Fig. 4b). The C concentration in stems, senescent leaves

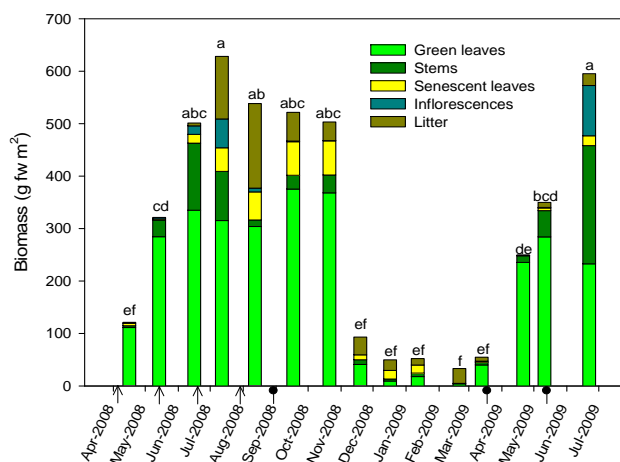


Fig. 2. Seasonal variation in biomass of fresh matter of different tissues of ryegrass. Arrows represent N application as NH_4NO_3 ; the bar with solid circle represents N application as urea. Values are means of 4 replicates (2 in each of the North and South fields). Different letters indicate significant differences at $P < 0.05$.

and inflorescences was lower than that in green leaves and attained larger seasonal variation, decreasing down to about 31 % during winter.

The C/N ratio in green leaves was relatively constant around 10 until spring 2009 when it increased to about 15 (Fig. 4c). The other tissues had higher and more variable C/N ratio with a peak of about 20–30 in summer 2008 and relatively larger values in spring 2009 compared to spring 2008.

3.4 Bulk tissue NH_4^+ and Γ (NH_4^+/H^+ ratio)

High NH_4^+ concentrations were recorded in senescent leaves (Fig. 5). All tissues showed distinct seasonality with peaks in NH_4^+ concentration after mineral fertilizer application. In addition, relative high concentrations occurred in winter 2009 several months after the last application of nitrogen fertilizer (urea) had taken place by the end of August 2008. Ammonium concentrations decreased steadily during spring and summer in 2009, even after urea input (Fig. 3).

Bulk tissue Γ , i.e. ratio between bulk tissue concentrations of NH_4^+ and H^+ , was constructed as a simple indicator for a comparison of NH_3 exchange potential among different tissues. The largest Γ value was present in senescent leaves (Fig. 4c) reflecting both high pH and NH_4^+ level. Litter also showed large values of Γ . Mixed senescent leaves and litter have previously been demonstrated to act as an NH_3 source (Mattsson et al., 2009). The seasonal variations in Γ values paralleled that of bulk tissue NH_4^+ rather than that of pH.

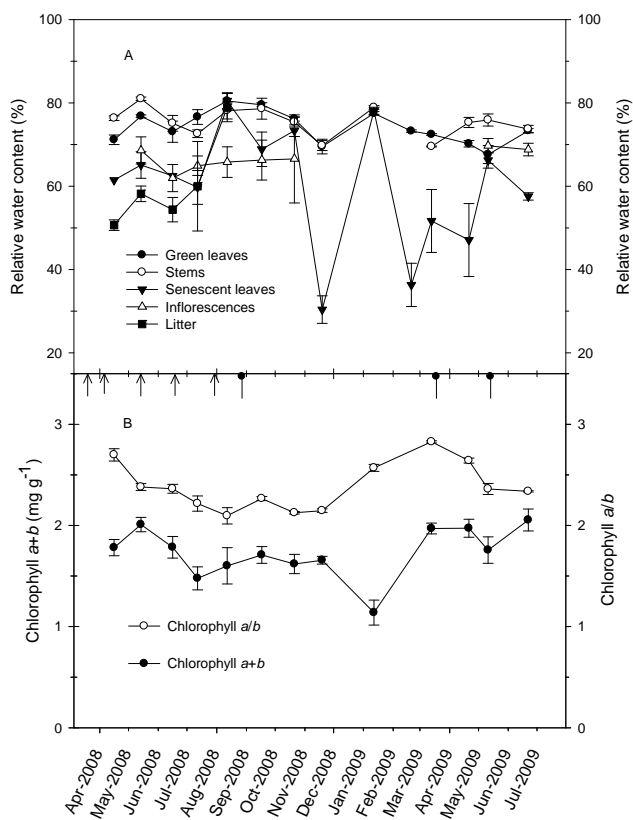


Fig. 3. Seasonal variations in tissue relative water content (A) and leaf chlorophyll and chlorophyll *a/b* ratio (B) in different tissues of ryegrass. Arrows below the X-axis represent N application as NH_4NO_3 ; the bar with solid circle represents N application as urea. Values are means \pm SE ($n = 4$).

3.5 Bulk tissue NO_3^- and total soluble nitrogen in ryegrass

Bulk tissue NO_3^- concentration showed clear peaks after mineral fertilizer application in 2008. Relatively high concentrations remained some time after the last application of urea in August 2008 followed by a gradual decline to a low level that was stable throughout 2009, not even responding to fertilizer application (Fig. 6a). The total soluble N concentration (substrate N) reflects a dynamic N pool available for growth. It constituted 16–34% of total N in green leaves and 24–42% of total N in stems. As expected, substrate N was relatively low in senescent leaves although with a peak in May–June 2008 (Fig. 6b), paralleling the high N concentration in these two months (Fig. 4a). After March 2009, substrate N gradually declined in all tissues.

3.6 Grass seasonal abundances of ^{15}N ($\delta^{15}\text{N}$) and ^{13}C ($\delta^{13}\text{C}$)

All tissues of ryegrass showed similar seasonal pattern of $\delta^{13}\text{C}$ (Fig. 7a). During spring 2008, $\delta^{13}\text{C}$ increased and a

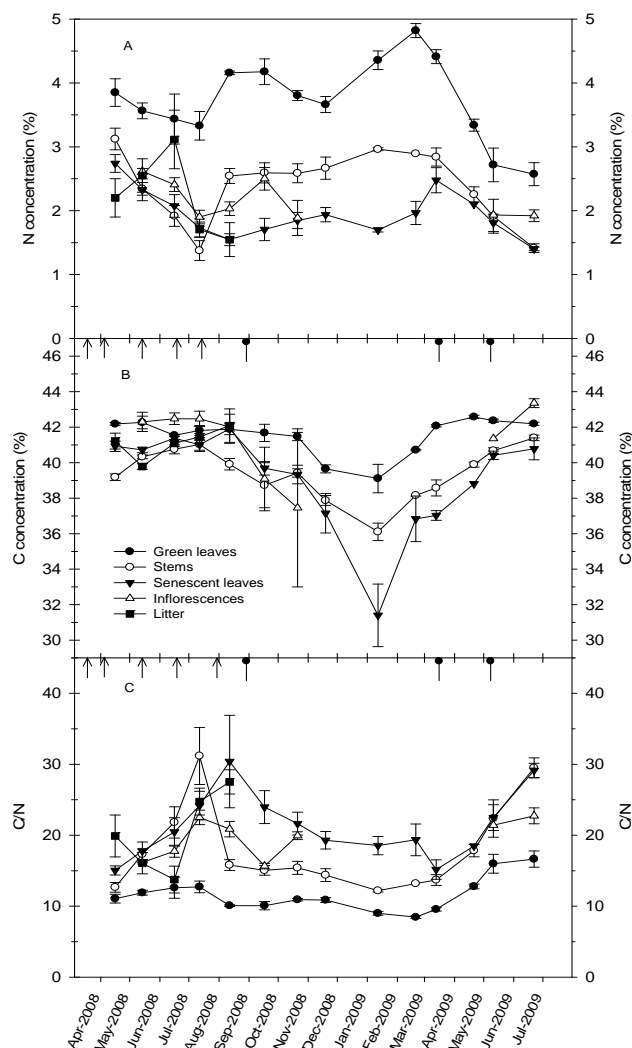


Fig. 4. Seasonal variation in total N concentration (A), total C concentration (B) and C/N ratio (C) per unit dry matter of different tissues of ryegrass. Arrows below the X-axis represent N application as NH_4NO_3 ; the bar with solid circle represents N application as urea.

peak value around -27.5‰ was reached in summer 2008. Thereafter $\delta^{13}\text{C}$ gradually decreased to a more negative value (-30 to -32‰) which lasted until early spring 2009 when $\delta^{13}\text{C}$ increased again. Green leaves had more negative $\delta^{13}\text{C}$ values than the other tissues. The seasonal pattern of $\delta^{15}\text{N}$ varied oppositely to that of $\delta^{13}\text{C}$, i.e. with a decrease during spring in both years and an increase during autumn and winter. In periods with intensive N application (April–July of both years), the $\delta^{15}\text{N}$ values declined in all tissues and green leaves obtained a similar $\delta^{15}\text{N}$ value as senescent leaves (Fig. 7b).

Table 2. Correlation coefficients (r) for key parameters in different ryegrass tissues. Numbers in bold in the lower left part of the table are r values for green leaves, while numbers in the upper right part are r values for stems. Correlation analyses were based on Pearson's product moment if the data met the normality requirements in the Shapiro-Wilk test; otherwise Spearman's rank order was used. * and ** denote that r values are significantly different from 0 at the 95 % and 99 % confidence levels, respectively.

	[C]	[N]	C/N	$[\text{NH}_4^+]$	pH	Γ	$[\text{N}]_{\text{substrate}}$	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$	Stems
[C]	–	–0.82**	0.92**	–0.06	0.31	–0.03	–0.65*	0.82**	–0.73**	[C]
[N]	–0.54*	–	–1.00**	0.09	–0.44	–0.03	0.86**	–0.80**	0.74**	[N]
C/N	0.68**	–0.98**	–	–0.17	–0.04	–0.13	–0.81**	0.69**	–0.82**	C/N
$[\text{NH}_4^+]$	–0.42	0.28	–0.35	–	–0.38	0.97**	0.10	0.15	–0.07	$[\text{NH}_4^+]$
pH	–0.10	0.19	–0.20	0.10	–	–0.21	–0.26	0.50	–0.32	pH
Γ	–0.54*	0.39	–0.45	0.91**	0.47	–	0.07	0.27	–0.12	Γ
$[\text{N}]_{\text{substrate}}$	–0.44	0.67**	–0.71**	0.02	0.29	0.21	–	–0.56*	0.64*	$[\text{N}]_{\text{substrate}}$
$\delta^{13}\text{C}$	0.65*	–0.71**	0.69**	–0.17	–0.17	–0.31	–0.31	–	–0.62*	$\delta^{13}\text{C}$
$\delta^{15}\text{N}$	–0.33	0.68**	–0.66**	0.20	0.08	0.26	0.25	–0.81**	–	$\delta^{15}\text{N}$
Green leaves	[C]	[N]	C/N	$[\text{NH}_4^+]$	pH	Γ	$[\text{N}]_{\text{substrate}}$	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$	

3.7 Relationship between nitrogen pools and climatic conditions

The $\delta^{15}\text{N}$ values in green leaves and stems were strongly positively correlated with the total N concentration ([N]; Table 2). The same was the case for the soluble N concentration ($[\text{N}]_{\text{substrate}}$), while $\delta^{13}\text{C}$ correlated negatively with [N] in both leaves and stems. No significant correlation was observed between $[\text{NH}_4^+]$ and [N] (Table 2). $\delta^{15}\text{N}$ values in green leaves, stems and senescent leaves were negatively correlated with the mean monthly temperature, while the corresponding $\delta^{13}\text{C}$ values were positively correlated (Fig. 8). The abundance of the stable isotopes showed no significant correlation with the accumulated monthly precipitation except for $\delta^{13}\text{C}$ in senescent leaves where there was a weak positive relation (Fig. 8).

4 Discussion

4.1 Grass seasonal growth

The temperate climate at the experimental site used in the present investigation provided optimum conditions for ryegrass growth as ample rainfall generally coincided with high temperature (Fig. 1). Ryegrass is a perennial species showing clear seasonality in its growth. The aboveground biomass accumulated rapidly between spring and summer and remained at a relatively high level until late autumn. Most of the management practices, including grazing and fertilization, were occurring in this period. However, despite few grazing events during late-autumn and winter, the biomass declined to a low level. Similar growth pattern of ryegrass have been shown in previous studies (Thomas and Norris, 1979; Brereton and McGilloway, 1999) and the slow growth rate in winter was related to the low temperature (Brereton and McGilloway, 1999). During late autumn and winter, senescent leaves and

litter accounted for a large proportion of the aboveground biomass, while outside this period green leaves dominated the ryegrass canopy (Fig. 2). The inflorescences became visible in May and developed to maturity during the following months with high temperature and precipitation.

The relative water content in green organs did not show large variations, while that in senescent tissues varied considerably depending on the precipitation several days before sampling (Fig. 3a). This reflects that the green organs of the grass were able to regulate their water status, while this property was lost during senescence. Chlorophyll and the chlorophyll a/b ratio decreased during senescence in the perennial ryegrass plants (Fig. 3b) as is the case in annual plant species and deciduous trees (Kurahotta et al., 1987; Wang et al., 2011). In perennial species, green leaves are more persistent and continuously emerge although at greatly reduced rate during the winter (Thomas and Norris, 1979). The increase in chlorophyll a/b ratio during winter coincided with decreasing total chlorophyll content and may partly be attributed to increasing ratio of old versus new leaves (Fig. 2) and partly to faster degradation rates of chlorophyll b , which constitutes a relatively high proportion of the outer parts of the antenna in the light-harvesting complexes, while chlorophyll a is more abundant in the reaction centre of the photosystem (Kurahotta et al., 1987).

4.2 Grass seasonal N status and turnover

The experimental grassland sites used in the present study were dominated by rye-grass and were important pastures for sheep and lamb grazing. A large amount of N-fertilizer was applied to the soil in order to increase biomass production. The N-fertilizer was split over 5 dressings in the period April–September 2008 and 2 in March–June 2009 (Fig. 1). A large seasonal decline in tissue N was therefore not expected. However, all plant tissues showed declining N concentration from spring to summer (April–July; Fig. 3) along with rapid

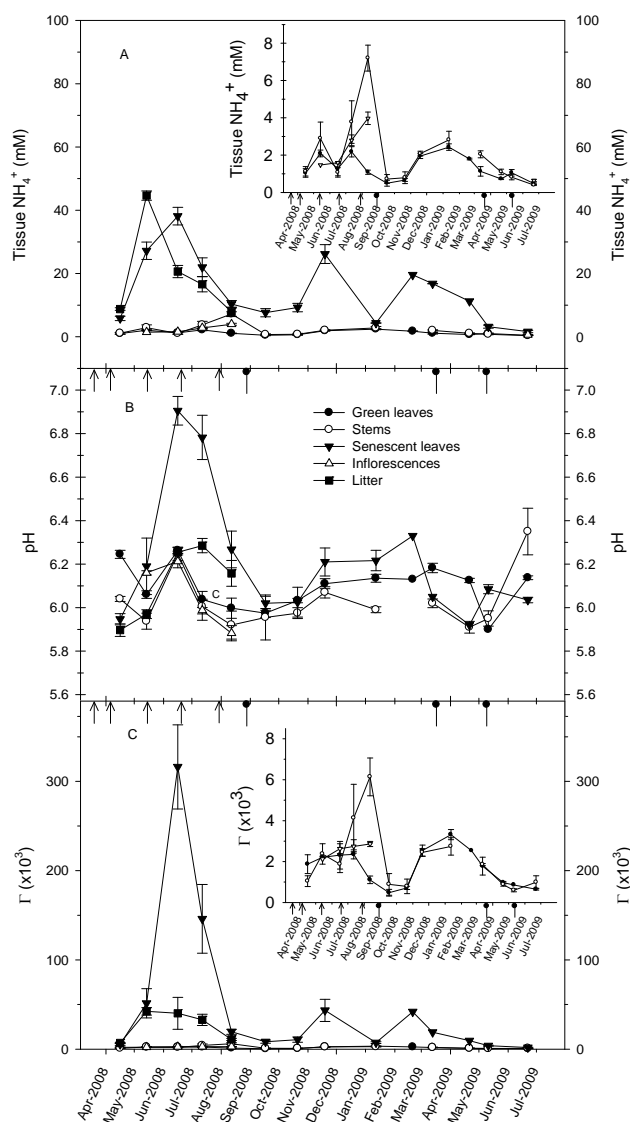


Fig. 5. Seasonal variations in bulk tissue NH_4^+ concentration (A), pH (B) and Γ (C) in different tissues of ryegrass. Inserts in A and C show bulk tissue NH_4^+ concentration and Γ , respectively, in green leaves, stems and inflorescences at a different scaling in order to reveal the fluctuations. Arrows below the X-axis represent N application as NH_4NO_3 ; the bar with solid circle represents N application as urea.

increase in biomass (Fig. 2), indicating the large demand for N in this period. A similar decline in N was observed despite high spring N fertilizer input in grassland in The Netherlands (van Hove et al., 2002). Plants sampled during the winter (January and February) showed increasing N concentration in green leaves as well as high N status of the other tissues (Fig. 3; see also van Hove et al., 2002). At low winter temperatures, the photosynthesis of grassland is low even at high foliar N concentrations (Skinner, 2007). The reduced C fixation results in decreasing C concentration of ryegrass tissues

in winter (Fig. 4). In general, green leaves had the largest N concentration followed by stems and inflorescences, and the senescent leaves had the lowest concentration, indicating N reallocation from the senescing leaves.

The seasonal variation of bulk tissue NH_4^+ concentrations in green leaves ranged from 0.5 to 2.5 mM (Fig. 5). These values are in the range previously reported for ryegrass before fertilization, but much lower than the peak values recorded within a few days after cutting/fertilization by Loubet et al. (2002) and Mattsson et al. (2009). In the present study, ryegrass was in all cases except one sampled more than 10 days after fertilization which may explain the lower levels of NH_4^+ in the green leaves. The seasonal variation in NH_4^+ concentration in stems and inflorescences was similar to that of green leaves, showing peaks in spring-summer and winter (Fig. 5). The senescent leaves and litter always had the largest NH_4^+ concentration ranging from about 2 to 45 mM, which was more than 10 times lower than peak value after cutting/fertilization reported by Mattsson et al. (2009).

The NO_3^- concentration in all plant parts except inflorescences increased many-fold during intensive N application periods in spring 2008 and thereafter declined during the autumn (Fig. 6), reflecting that the uptake of NO_3^- exceeded the assimilation at high soil N availabilities (Ourry, 1989; Whitehead, 1995). Total soluble N constituted 20–25 % of total N (compare Fig. 6b and 4A) and there was strong positive correlation between the two parameters in all plant tissues except inflorescences (Table 2). All N pools were much lower in spring 2009 compared to 2008. This difference may reflect that the precipitation from mid March until end of June in 2009 was only 35 mm against 96 mm in 2008. In addition, the fertilizer applied in 2009 was urea as opposed to 2008 when it was NH_4NO_3 . Due to the low precipitation, a considerable part of the urea applied in 2009 may not have become available for the plants or may have been lost by NH_3 volatilization because the fertilizer was not dissolved into the soil profile but remained on the surface (Schjoerring and Mattsson, 2001; Sommer et al., 2004).

4.3 Grass seasonal NH_3 exchange potential

Episodes of NH_3 emission from grasslands have frequently been observed in response to cutting (Sutton et al., 1998, 2001; David et al., 2009; Mattsson et al., 2009; Milford et al., 2009). Nitrogen fertilization also gives rise to NH_3 emission (Bussink et al., 1996; Larsson et al., 1998; Herrmann et al., 2001; Ross and Jarvis, 2001; Sutton et al., 2001; David et al., 2009; Mattsson et al., 2009; Milford et al., 2009; Flechard et al., 2010; Hojito et al., 2010). The magnitude of NH_3 emission following fertilization may be much larger than that after cutting (Milford et al., 2009). The driver for NH_3 exchange between plants and the atmosphere is the stomatal compensation point for NH_3 , reflecting external N availability and internal plant processes involved in N assimilation and turnover (Mattsson and Schjoerring, 1996; Mattsson et

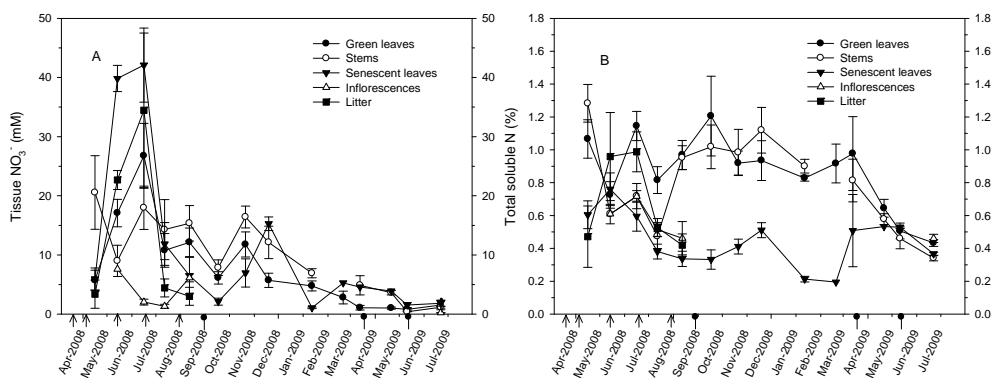


Fig. 6. Seasonal variations in bulk tissue NO_3^- concentration (A) and total soluble N (B) per unit dry matter in different tissues of ryegrass. Arrows below the X-axis represent N application as NH_4NO_3 ; the bar with solid circle represents N application as urea.

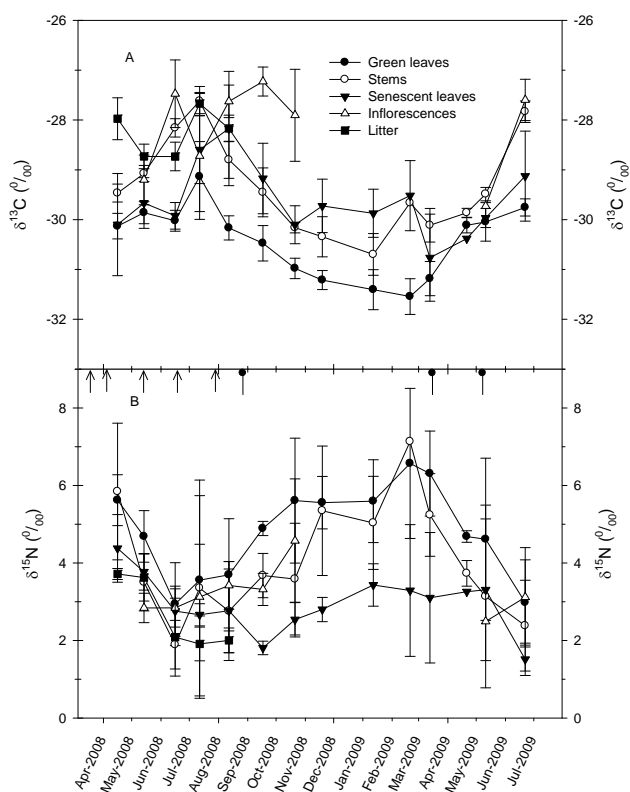


Fig. 7. Seasonal variations in $\delta^{13}\text{C}$ (A) and $\delta^{15}\text{N}$ (B) in different tissues of ryegrass.

al., 1998; Husted et al., 2000b; Massad et al., 2008). The stomatal compensation point for NH_3 is a function of leaf temperature and the concentrations of NH_4^+ and H^+ in the leaf apoplastic solution. The ratio between the NH_4^+ and H^+ concentrations is designated the stomatal Γ value. In the absence of stomatal Γ values, bulk tissue Γ can be considered as a potential NH_3 exchange indicator for ryegrass (Mattsson and Schjoerring, 2002; David et al., 2009; Mattsson et

al., 2009) although the parameter is not a direct measure of the NH_3 exchange between plant and atmosphere.

In the present study, the bulk Γ values had a similar seasonal pattern in all the different tissues of the ryegrass plants (Fig. 5c). Relatively high Γ values were observed not only during the summer period with intensive fertilization and grazing events, but also in some cases during the winter (Fig. 5c). Most of the Γ values in the green plant parts were <3000 , which is consistent with values up to 2600 (David et al., 2009) and 2700–3800 (Mattsson et al., 2009) previously reported for intensively managed grassland and also with a canopy Γ value of around 2200 in a non-fertilised grassland (Kruit et al., 2007). In these ranges of Γ values, green leaves are clearly sinks for NH_3 (David et al., 2009; Herrmann et al. 2009). Senescent leaves in all cases had much higher Γ values than green plant parts (Fig. 5c). During periods with intensive N input, both the NH_4^+ concentration and the pH increased, resulting in a very high Γ value of 320 000 (Fig. 5c). This indicates a large potential for NH_3 emission in agreement with that senescent leaves and litter are important NH_3 sources (Mattsson and Schjoerring, 2003; David et al., 2009; Herrmann et al., 2009). Harper et al. (2000) suggested that NH_3 emission from foliage may persist over prolonged periods, most of the time occurring from the surface rather than via the stomata. In the present study, the Γ values decreased rapidly after management events and only relatively small and short increases occurred during late autumn and winter due to high NH_4^+ concentrations rather than high pH (Fig. 5). Increasing tissue NH_4^+ during winter may reflect lack of C skeletons required to assimilate NH_4^+ due to low photosynthesis (Finnemann and Schjoerring, 1999). Γ peak values coincided in green and senescent leaves (Fig. 5) and one of the possible reason is that the green leaves recaptured the NH_3 released by senescent leaves and litter (David et al., 2009; Mattsson et al., 2009), which resulted in increased NH_4^+ concentration.

Coinciding micrometeorological measurements showed that NH_3 emission dominated over the grassland during the

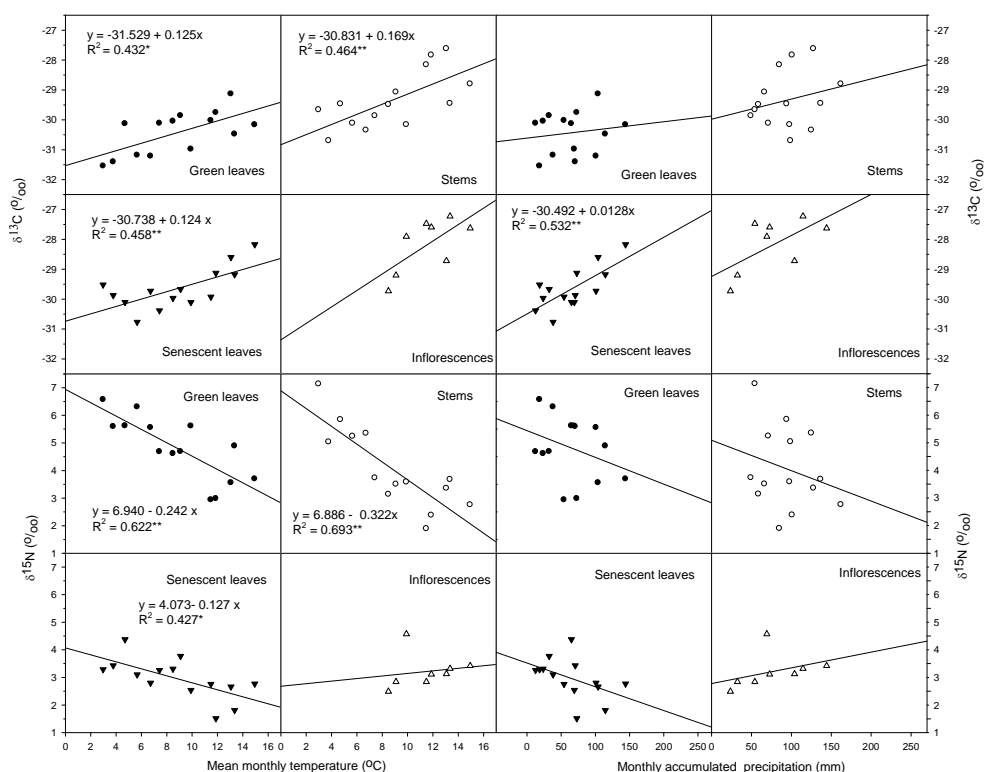


Fig. 8. Regression analysis of foliar natural abundance of stable isotopes in different ryegrass tissues ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) in relation to climatic conditions (mean monthly temperature and accumulated monthly precipitation over a 30 days period prior to sampling). The regression equation is shown only in cases where a significant correlation was observed.

second half of August until beginning of September 2008 (data not shown). The Γ values in the senescent leaves and litter were very high during the preceding summer period, but had declined by mid August (Fig. 5). This suggests that the main source of NH_3 in the second half of August was mineral fertilizer. Urea application by the end of August 2008 caused an immediate large NH_3 emission, which rapidly declined after a few days due to rainfall (data not shown).

4.4 Changes in natural abundance of ^{13}C and ^{15}N

Soil $\delta^{15}\text{N}$ values are generally higher relative to plant and atmospheric values (Amundson et al., 2003). This reflects that soil microbes discriminate against the heavy isotope during processes such as mineralization, nitrification, denitrification and NH_3 volatilization (Watzka et al., 2006). Grazers may intensify losses of ^{15}N -depleted products from soils, thereby leading to further increase in soil $\delta^{15}\text{N}$ (Frank and Evans, 1997). Mineral N fertilizers have been reported to have low $\delta^{15}\text{N}$ values, ranging from at -1.4 to 2.6‰ in NH_4NO_3 and -5.9 to -0.8‰ in urea (Bateman and Kelly, 2007). Accordingly, $\delta^{15}\text{N}$ values decreased during spring and summer when fertilizers were applied (Fig. 7b). During periods when absorbed N mainly derives from mineralization of organic N, $\delta^{15}\text{N}$ values will increase (Gebauer et al., 1994; Hogberg,

1997; Craine et al., 2009b) as was also the case during autumn and winter in the present work (Fig. 7b). There was a clear difference among different plant parts in $\delta^{15}\text{N}$ values during autumn and winter, while values recorded during fertilizer application in spring and summer were much more uniform (Fig. 7b). During autumn and winter, green leaves showed higher $\delta^{15}\text{N}$ values than stems and with senescing leaves having the lowest ^{15}N abundance (Fig. 7b). This is quite remarkable as the biomass was declining during the same period (Fig. 2) but must reflect that the absorbed N originated from mineralization and primarily was allocated to the green leaves, the latter also supported by the fact that their total N concentration was the highest (Fig. 4a).

The N availability influences plant growth and thus the C status in the grassland (Ammann et al., 2009). The $\delta^{13}\text{C}$ variation pattern for ryegrass observed in the present study (Fig. 7a) indicates less discrimination against ^{13}C during reproductive compared to vegetative growth, but the seasonal pattern may vary between species (Smedley et al., 1991). During intensive N application (April–July of both years), the $\delta^{13}\text{C}$ values were constant or slightly elevated in all tissues (Fig. 7a). This was a period with intensive N uptake and growth which will have stimulated photosynthesis, leading to lower internal CO_2 concentrations and more efficient use of the heavy ^{13}C isotope, thereby reducing the discrimination

and leading to less negative $\delta^{13}\text{C}$ values (Farquhar et al., 1989; Stamatiadis et al., 2007). During period with less active growth, i.e. during autumn and winter, photosynthesis is less efficient, leading to increased discrimination and more negative $\delta^{13}\text{C}$ values (Fig. 7a). Water availability also influences this relation via affecting stomatal conductance and hence the internal CO_2 concentration, leading to less negative foliar $\delta^{13}\text{C}$ at low water availability (Denton et al. 2001; Brüggemann et al., 2011).

Several studies have related the foliar natural isotopic abundance to climatic conditions and nutrient concentration in leaves. At a global scale, Amundson et al. (2003) and Craine et al. (2009b) reported a negative relationship between foliar $\delta^{15}\text{N}$ and mean annual precipitation, while foliar $\delta^{15}\text{N}$ was positively correlated with the mean annual temperature ($\geq -0.5^\circ\text{C}$) at undisturbed sites. Mineralization of soil organic matter is expected to increase with soil temperature, leading to increased $\delta^{15}\text{N}$ values. The negative correlation between $\delta^{15}\text{N}$ and temperature in not only green leaves but also in stems and senescent leaves observed in the present study (Fig. 8) shows that other factors than soil N mineralization, e.g. fertilizer application, affected $\delta^{15}\text{N}$. Based on analysis of 24 plant species in central North American grasslands during thirteen decades, McLauchlan et al. (2010) deduced that foliar $\delta^{15}\text{N}$ increased with foliar N concentration. Craine et al. (2009a) observed a similar positive correlation in 49 grass species across 330 South African savannah sites. Similar to these large-scale observations, $\delta^{15}\text{N}$ and N concentration in green leaves, stems and senescent leaves were positively correlated in the present study (data not shown).

In terms of $\delta^{13}\text{C}$, Li et al. (2005) reported a positive relation between foliar $\delta^{13}\text{C}$ and mean monthly temperature or monthly accumulated precipitation in four tree species. Similar data does not exist for grasslands. A significant positive relation between mean monthly temperature and $\delta^{13}\text{C}$ in green leaves, stems and senescent ryegrass leaves was observed in the present work (Fig. 8), but there was no relation with monthly accumulated precipitation except for senescent leaves which showed a positive correlation (Fig. 8).

5 Conclusions

The seasonal pattern of the Γ value, i.e. the ratio between NH_4^+ and H^+ concentration, was similar for various tissues of ryegrass plants although the relative magnitude of Γ differed. Green leaves and stems generally had low Γ values, indicating that they were sinks for atmospheric NH_3 . However, substantial peaks in Γ occurred in green leaves and stems during spring and summer in response to intensive fertilization and grazing events. Compared to green tissues, Γ in senescent leaves and litter were much higher, suggesting a large potential for NH_3 emission. Peaks in Γ occurred during the winter. These peaks may reflect fluctuations in soil N availability and plant N assimilation capacity as evidenced by

changes in the abundance of the stable isotopes ^{15}N and ^{13}C . During autumn and winter, $\delta^{15}\text{N}$ values increased, indicating that the ryegrass plants absorbed N derived from mineralization of soil organic matter. At the same time, $\delta^{13}\text{C}$ values decreased, pointing to limitations in photosynthesis, which may have reduced the capacity for N assimilation. Temperature had a greater influence than precipitation on seasonal changes in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ in the ryegrass plants.

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