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Allocation patterns of airborne nitrogen in mountainous heathlands – A ^{15}N tracer study in the Cantabrian Mountains (NW Spain)

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ABSTRACT

The fate of atmospheric N depositions in heathlands dominated by *Calluna vulgaris* (L.) Hull in the Cantabrian Mountains (NW Spain) was analyzed in this study. The aim was to identify and quantify allocation patterns of airborne nitrogen in mountain heathland ecosystems by ^{15}N tracer experiment. Four replicated plots were established to analyze ^{15}N partitioning among different compartments selected (*Calluna* biomass, soil horizons and soil microbial biomass), besides N losses by leaching, using ^{15}N tracer pulse addition. The study was conducted over two years. The recovery of ^{15}N tracer was significantly higher (72%) in the first year compared to the second year (5%). Most ^{15}N was recovered in the soil compartment in both years, mainly in the O-horizon. ^{15}N losses by leaching were negligible over two years, suggesting that the ecosystem was not N saturated. Low ^{15}N tracer recovery was found both in the new shoots of *Calluna* (0.5%) and the old ones (1.3%) in the short-term. The soil microbial biomass was not an important N sink in these heathlands. This study demonstrates that Cantabrian heathlands have a capacity to immobilize nitrogen in a short time, but that N is transferred out of the ecosystem during the second year.

1. Introduction

The emission of reactive nitrogen (N) and its accumulation in ecosystems and environmental reservoirs have increased dramatically over the past 100 years (Galloway et al., 2004, 2008). This has contributed to significant shifts in global N cycles, currently one of the most pressing problems in ecosystem and biodiversity protection (Erisman et al., 2011; Sutton et al., 2012). For example, it is estimated that 40% of the world's protected areas will receive atmospheric N loads above 10 kg N ha^{-1} year^{-1} in 2030 (Bleeker et al., 2011), with consequences for the functioning of ecosystems and the services they provide (Bobbink et al., 2010). From a European perspective the highest rates of N deposition currently occur in the centre of the continent, with a range of 30–50 kg N ha^{-1} year^{-1} (Simpson et al., 2006).

The Cantabrian Mountains (NW Spain) have been recognized as a biodiversity hotspot, hosting a wide variety of ecosystems and endemic species (Morán-Ordóñez et al., 2011). However, the Cantabrian Mountains are also affected by atmospheric inputs of N, ranging between 7.5 and 15 kg N ha^{-1} year^{-1} (García-Gómez et al., 2014). Such input rates are considered as critical loads with regard to the long-term preservation of ecosystem functions and the biodiversity typical of the Cantabrian Mountains (Bobbink et al., 2010). N accumulation has been shown to cause declines in biodiversity via the expansion of nitrophilous species and the competitive exclusion of others (Calvo et al., 2005, 2007; Ariño et al., 2011; Southon et al., 2013); nutrient imbalances in plant tissues and soil acidification (De Graaf et al., 2009; Stevens et al., 2011); changes in community composition (Clark et al., 2013); and increasing susceptibility of plants to secondary stressors (such as herbivory; Power et al., 1998).

One of the most representative ecosystems in the Cantabrian Mountains is *Calluna-vulgaris*-heathland, which also represents the southern distribution range of heathlands in Europe (Loidi et al., 2010; Fagúndez, 2013). *Calluna*-heathlands are low-N environments (Edmondson et al., 2010; Friedrich et al., 2011b), and increasing N availability can lead to severe shifts in their functioning (Vitousek et al., 1997). These effects are related to the quality and quantity of N retained in soil and vegetation, the release from internal N cycles, and N losses from the soil as gas flow and leachate (Phoenix et al., 2012). Some ecosystems with high and long-term N inputs achieved N saturation (Gao et al., 2014) resulting

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in a reduced ability to further store larger N amounts in soils and plant biomass, and in N losses to the groundwater and atmosphere (Vitousek et al., 1997; Britton et al., 2008). Although there is evidence about the quantity and dispersion of emitted N into the atmosphere, little is known about the fate of airborne N loads within ecosystems (Galloway et al., 2004). In the mid-90s, only 35% of the N deposited in ecosystems could be traced, with uncertainties about the fate of the remaining 65% (Galloway et al., 2008). Field studies with experimental manipulations of N inputs showed that European heathlands are able to retain 60–90% of the N supplied, even with high fertilization rates (Pilkington et al., 2005b). Most of the N retained was found in the soil, mainly in the organic horizon (Power et al., 1998; Pilkington et al., 2005b), although *Calluna* biomass immobilized a significant proportion of added N (Carroll et al., 1999; Friedrich et al., 2011b). In the organic soil horizons, microbial biomass is also able to incorporate N from atmospheric inputs (approximately two-thirds of total N in the ecosystem; Phoenix et al., 2012), resulting in increasing microbial activity (Power et al., 2006) and decreasing C:N ratios (Perakis et al., 2005). N losses via leaching are highly variable, depending on the type of ecosystem and study area, as this process is controlled by parameters such as N deposition rates, the exchange and storage capacity of the humus horizons, the nutrient uptake by plants and soil microbes, and internal nutrient cycles (Pilkington et al., 2005a; Härdtle et al., 2007).

Field studies with an experimental manipulation of N inputs may evaluate N storage patterns in ecosystems, but do not allow for a quantification of fluxes (Southon et al., 2013). Thus, from a functional point of view, one of the most adequate procedures to identify allocation or partitioning patterns of airborne N in ecosystems is the use of ^{15}N tracer (Robinson, 2001; Schlesinger, 2009). Stable isotopes have the advantage of being present in nature, and changes in distribution and abundance in plants and soils provide relevant information related to different functions of an ecosystem (Michener and Lajtha, 2007). In tracer studies, ^{15}N isotope signatures provide information about the N cycle, N input/output flows and thus, N budgets or the storage capacity for N in different ecosystem compartments (Carreira et al., 2010). Recent studies with ^{15}N tracer (e.g. in forest ecosystems) showed that soil compartments, mainly humus horizons, acted as a major sink for airborne N (Tietema et al., 1998; Nadelhoffer et al., 2004; Sheng et al., 2014). In addition, the soil microbial biomass of the organic layer was found to immobilize 50–75% of the ^{15}N recovered in the humus fraction (Tye et al., 2005). In some heathlands, the bryophyte layer proved to be an important short-term sink for N, accounting for 40–65% of recovered ^{15}N tracer (Curtis et al., 2005; Friedrich et al., 2011a). However, there are no studies about N allocation patterns of mountain heaths, particularly the heathlands of the Cantabrian Mountains. Cantabrian heathlands exhibit highly specific climatic conditions, since they represent an ecotone between an Atlantic and Mediterranean climate, and are characterized by soil types that differ from those of heaths typical of Central and northern Europe (Fagúndez, 2013). These differences may influence both the sequestration and allocation of airborne N (Galloway et al., 2008; Jones and Power, 2012; Templer et al., 2012; Southon et al., 2013). Recent N fertilization experiments showed that *Calluna* plants and soils are capable of sequestering and immobilizing high amounts of N despite current rates of atmospheric N inputs (Marcos et al., 2003; Villalón, 2014). So we expect Cantabrian heathlands to have large N storage capacities in both soil and aboveground biomass (Aber et al., 1998; Templer et al., 2012).

The aim of this study was to quantify N sequestration and allocation patterns in Cantabrian heathlands using ^{15}N as a tracer. We analyzed the fate of experimentally added tracer in different ecosystem compartments (vegetation and soil, including soil microbial biomass), and quantified ecosystem N losses via leaching. We hypothesized that Cantabrian heathlands are still not N saturated (despite current atmospheric inputs), indicated by high N sequestration rates and low N losses via leaching.

2. Materials and methods

2.1. Study site

The study site is located on the León (south) side of the Cantabrian Mountain range (NW Spain). Two representative and homogeneous *Calluna* heathland sites were selected, situated 90 km apart from each other. San Isidro (1636 m.a.s.l., 43°03′N, 5°21′W) represents a flat, continuous heathland area facing north and exposed to winds. La Majúa (1770 m.a.s.l., 43°01′N, 6°05′W) is north-facing with a small slope. These sites have a Eurosiberian climate that is characterized by a dry period of less than 2 months in summer and a snow cover in winter which remains until the end of May. The length of the growing season in Cantabrian Mountains ranges from May to October. The highest rates of precipitation during the growing season occur in late-autumn (October), mainly as snowfall, while the lowest rates were recorded in late-spring and summer months. Mean annual temperature is 5.5 °C. The soil is an Umbrisol (European Commission, 2005), with a depth of about 45 cm (on sandstone and lutite). These soils are sandy, very acidic and have low fertility. The study sites are characterized by homogeneous patches of prevailing *C. vulgaris* (>75% of cover). Accompanying species are *Erica tetralix*, *Vaccinium myrtillus*, and other grass and forb species up to 15% cover (Calvo et al., 2005), mainly *Nardus stricta*, *Juncus squarrosus* and *Deschampsia flexuosa*. The bryophyte cover in these heathlands is below 1%.

2.2. Study design

Two plots of 3 m × 7 m were randomly selected at each study site in homogenous stands of *Calluna* in July 2011 (i.e. four replicates in total). Two 1 m × 2 m subplots were fixed within each plot, one of which received ^{15}N tracer (henceforth referred to as 'labelled subplot'), and the other was used to determine the natural abundance of ^{15}N (henceforth referred to as 'control subplot'). Both subplots were separated by a distance of 1 m to avoid contamination of the control subplot. To calculate N losses by leaching, other four 3 m × 5 m plots were established in both study areas (two in San Isidro and two in La Majúa). Within each plot two lysimeters were installed, one received ^{15}N tracer ('labelled lysimeter'), and the other was used to measure the natural abundance of ^{15}N ('control lysimeter'). One of the lysimeters from La Majúa was broken during the second month of the experiment, so we finally used three replicates for leaching measurements.

The lysimeter consisted of a PVC pipe (40 cm length and 50 cm diameter) and was slowly hammered into the soil. The surrounding soil was removed consecutively, so that the pipe finally contained an undisturbed soil core covered by *Calluna*. The bottom end of the pipe was then sealed and made air-tight with a PVC lid (with outlets for seepage water connected to a pump), and subsequently buried at the same location. A porous disc (PE-sinter; ecoTech, Bonn, Germany) covered by a nylon membrane (pore diameter 0.45 μm; Whatman Ltd., Maidstone, UK) was installed at the bottom of each lysimeter. All the seepage water leached through the lysimeter was sampled by means of a tension-controlled pump (−90 mbar) and collected continuously in glass bottles. The electrical system was powered by a 12 V gel battery, protected inside a sealed box. Plots and lysimeters were fenced in to prevent damage by grazing animals.

2.3. ^{15}N tracer addition

In the first week of July 2011 pulse labelling with $^{15}NH_4{}^{15}NO_3$ (98 atom %) was performed in all labelled subplots and labelled lysimeters. The labelled subplots and lysimeters received 105.9 mg m^{-2} of $^{15}NH_4{}^{15}NO_3$ tracer dissolved in 500 ml distilled water. This quantity aimed at a target $\delta^{15}N = 100‰$ in *Calluna* and was too small to cause a 'fertilization effect'. ^{15}N addition was applied with a spray bottle equipped with a nozzle that allowed for an evenly distributed addition of the ^{15}N tracer to the surfaces. Control subplots and control lysimeters received (area related) the same amounts of water. During this procedure *Calluna* twigs were lifted to avoid uptake of ^{15}N by leaves.

2.4. Calluna biomass, soil horizons and soil microbial biomass sampling

From July 2011 to November 2012 the following ecosystem compartments were sampled: *Calluna* biomass as (i) the current year's shoots (henceforth referred to as 'new shoots') and (ii) 1–2 year old shoots (henceforth referred to as 'old shoots'), soil horizons (O-, A- and B-horizons) and soil microbial biomass. Samples were collected ten times during the two growing seasons (i.e. 1, 2, 4, 10, 14, 18, 54, 61, 65 and 70 weeks after ^{15}N tracer addition; for exact samplings dates see Table 1) in both labelled and control subplots on each occasion. Ten randomly chosen new *Calluna* shoots and ten randomly chosen old *Calluna* shoots were cut in each subplot, and bulked to one sample for new and old shoots separately. Soil samples were collected in each soil horizon (O, A and B) per subplot using soil cores of 5 cm diameter. To analyze the soil microbial biomass 5 cm × 5 cm soil samples in the organic layer were taken in each subplot in the 10, 14, 18, 54, 61, 65 and 70 sampling weeks, coinciding with the vegetative activity period.

2.5. Leachate sampling

Leachate from lysimeters was collected continuously over the growing season. Samples from labelled and control lysimeters were taken at the same intervals as the biomass and soil samples. The total amount of extracted leaching was recorded in each lysimeter on each sampling date. During the winter months (from December to May) no sampling took place, because leachate was frozen in collecting flasks and covered by snow.

2.6. N contents and ^{15}N analysis in Calluna biomass, soil horizons, soil microbial biomass and leachate

Samples of *Calluna* shoots were dried at 40 °C for 48 h, ground with a mixer mill and sieved (200 μm) (Pulverisette 14, Fritsch, Oberstein, Germany). These samples were stored at room temperature in small glass capsules until analysis. Soil samples were air dried, ground and sieved (200 μm). The milled and sieved soil was stored under the same conditions as the biomass samples. Total C, N and $\delta^{15}N$ were determined using a continuous flow elemental analyser-isotopic ratio mass spectrometer (vario El cube, Elementar, Hanau, Germany, coupled to an Isoprime IRMS, Isoprime Ltd., Cheadle Hulme, UK) at the University of Lüneburg (Germany). To determine N content and $\delta^{15}N$ signatures of the soil microbial biomass we performed the extraction of cell N by the fumigation–extraction method (Brookes et al., 1985). N content was determined using a Kjeldahl digestion method. $\delta^{15}N$ signatures were determined by a diffusion method proposed by Stark and Hart (1996) and Sebilo et al. (2004). The diffusion filters were packed in tin capsules.

Leachate was filtered, and an aliquot was used immediately for analysis of $N{-}NH_4{}^+$ content using the salicylate method (Reardon et al., 1966). The remaining filtrate leachate was stored at −18 °C until thawed for analysis of $N{-}NO_3{}^-$ by ion chromatography. Modified diffusion method was used to determine $\delta^{15}N{-}NO_3{}^-$ in leachate samples (Sigman et al., 1997) and $\delta^{15}N{-}NH_4{}^+$ (Holmes et al., 1998). The filters obtained by the diffusion of leachate samples were packed in tin capsules. All filters (soil microbial biomass and leachate) were analyzed by the Stable Isotope Facility of the University of California (using an Elementar vario El cube, Elementar Analysensysteme, Hanau, Germany) interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) to analyze $\delta^{15}N$.

2.7. Calculations of N pools and N leaching losses

N pools were calculated by means of the pool masses multiplied by their N concentrations. To calculate the N pools in *Calluna* shoots, new and old shoots were cut from mature *Calluna* in an area of 0.25 m^2. Dry weight was determined after drying at 70 °C for 24 h. Total soil masses were determined considering the depth and bulk density of each horizon. N losses by leaching were calculated from the total amounts of leachate and their content of $N{-}NO_3{}^-$ and $N{-}NH_4{}^+$ in the labelled lysimeters.

2.8. Calculation of ^{15}N abundance, ^{15}N enrichment and ^{15}N tracer recover

^{15}N content of all samples was reported in $\delta^{15}N$ notation, which is the relative difference in $^{15}N/^{14}N$ ratios between samples and atmospheric N_2 (isotopically constant and designated by convention as 0‰). The $\delta^{15}N$ is calculated as a per mil (‰) using:

$$\delta^{15}N(‰) = [(R_{sample}/R_{standard}) - 1] \times 10^3 \quad (1)$$

where R is defined as the atomic $^{15}N/^{14}N$ ratio, and the standard is atmospheric N_2 (Fry, 2006). $\delta^{15}N$ values obtained from diffusion filters were used to calculate ^{15}N abundance in soil microbial biomass, and corrected with their respective $\delta^{15}N$ blank values according to the formula proposed by Stark and Hart (1996). A mass balance between fumigated $\delta^{15}N$ values and the corresponding non-fumigated $\delta^{15}N$ values was applied according to the formula used by Dijkstra et al. (2006). $\delta^{15}N{-}NO_3{}^-$ and $\delta^{15}N{-}NH_4{}^+$ values obtained from diffusion filters were used to calculate $\delta^{15}N$ signatures of the leachate, and corrected with their respective $\delta^{15}N$ blank values according to the procedure indicated in their diffusion protocols (Sigman et al., 1997; Holmes et al., 1998), followed by a mass balance between $\delta^{15}N{-}NO_3{}^-$ and $\delta^{15}N{-}NH_4{}^+$ for each leaching sample.

^{15}N enrichment in the ecosystem compartments was calculated using the formula given by Fry (2006), which expresses ^{15}N enrichment (‰) of a sample from a labelled subplot in relation to a sample from its respective control subplot.

^{15}N tracer recovery in each ecosystem compartment and ^{15}N leaching losses were calculated according to the formula proposed by Nadelhoffer et al. (2004). ^{15}N tracer recovery is expressed as % of ^{15}N tracer recovered mass in relation to total mass of ^{15}N tracer added to labelled subplots or labelled lysimeters:

$$^{15}N_{rec} = m_{pool} \times ((at.\%{}^{15}N_{pool} - at.\%{}^{15}N_{ref})/(at.\%{}^{15}N_{tracer} - at.\%{}^{15}N_{ref})) \quad (2)$$

where $^{15}N_{rec}$ is the mass of ^{15}N tracer recovered in the N pool of labelled subplots or in leachate losses from labelled lysimeters (g N m^{-2}), m_{pool} is the mass of the N pool of labelled subplots or the amount of total N leaching losses from labelled lysimeters (g N m^{-2}), at.%$^{15}N_{pool}$ is the at.%^{15}N in the N pool of labelled subplots or in leachate losses from labelled lysimeters, at.%$^{15}N_{ref}$ is the at.%^{15}N in the N pool of control subplots or in leachate from control lysimeters, and at.%$^{15}N_{tracer}$ is the at.%^{15}N of the added ^{15}N tracer.

2.9. Statistical analyses

Time differences between ^{15}N abundances in old/new *Calluna* shoots, soil horizons (O, A and B) and chemical compound of leaching (ammonia/nitrate) were tested using a three-way repeated measures ANOVA, with time as the repeated measure. ^{15}N abundance of soil microbial biomass in time was tested using two-way repeated measures ANOVA. To analyze the effects of ^{15}N enrichment in *Calluna* shoots, soil horizons and leaching losses in time, two-way repeated measures ANOVA was used with time as the repeated measure. The effect of ^{15}N enrichment in soil microbial biomass over time was tested using one-way repeated measures ANOVA. The differences in ^{15}N tracer recovery in each heathland compartment between the two years of study were tested using one-way repeated measures ANOVA, with time as the repeated measure. Besides, for each year, in order to determine differences in ^{15}N tracer recovery between new and old *Calluna* shoots and between soil horizons (O, A and B) we used a one-way ANOVA. Two reference dates were chosen (November 2011 and November 2012) to compare the performance of the system as a whole over time. Pearson correlation coefficients were also obtained for N content and ^{15}N tracer recovery of O-horizon, N content and ^{15}N tracer recovery of soil microbial biomass, and precipitation in order to examine potential relationships among factors. All statistical analyses were performed using SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. ^{15}N natural abundances in Calluna biomass, soil horizons and soil microbial biomass

^{15}N natural abundance (in terms of $\delta^{15}N$ signatures) in new and old *Calluna* shoots showed negative values, with means of −1.19‰ and −1.61‰, respectively (Table 1). ^{15}N natural abundance increased with soil depth at all sites and all sampling dates. O-horizon showed the highest variability in ^{15}N natural abundance (−0.45‰ and 4.41‰). In the A- and B-horizons ^{15}N natural abundance achieved values above 4‰ and 6‰, respectively. The highest mean values of $\delta^{15}N$ were found in the B-horizon (Table 1). ^{15}N natural abundance of the soil microbial biomass varied between −3.81‰ and 10.85‰, with no clear temporal pattern (Table 1).

3.2. ^{15}N enrichment in Calluna biomass, soil horizons and soil microbial biomass

All compartments of the labelled subplots were enriched in ^{15}N, with the lowest enrichment found in the B-horizon (Fig. 1). *Calluna* biomass (old and new shoots) showed a significant ^{15}N enrichment ($F_{(8,48)} = 4.137$, $p < 0.05$), particularly during the first two weeks after tracer application, but differences between old and new *Calluna* shoots were not significant. Furthermore, ^{15}N values peaked after 10 (new shoots) and 65 weeks (new and old shoots) following tracer application. ^{15}N enrichment decreased with increasing soil depth, with the highest values found in the O-horizon ($F_{(2,9)} = 22.453$, $p < 0.05$), and the lowest values in the B-horizon (Fig. 1). Values in soil horizons significantly increased in the first weeks of the experiment ($F_{(8,72)} = 8.115$, $p < 0.05$) with peaks in weeks 4 and 14 after tracer application, but then continuously decreased in subsequent weeks ($F_{(1,9)} = 31.100$, $p < 0.05$). This decrease was more pronounced in the O-horizon. Soil microbial biomass N was 0.89 g m^{-2} under natural conditions, equivalent to 4.24% of the total N in the O-horizon. ^{15}N enrichment in soil microbial biomass significantly increased during the first year of the experiment with a peak in week 54 ($F_{(2,9)} = 21.238$, $p < 0.05$), then dropped for 11 weeks (with a minimum in week 65), and achieved a second peek in week 70.

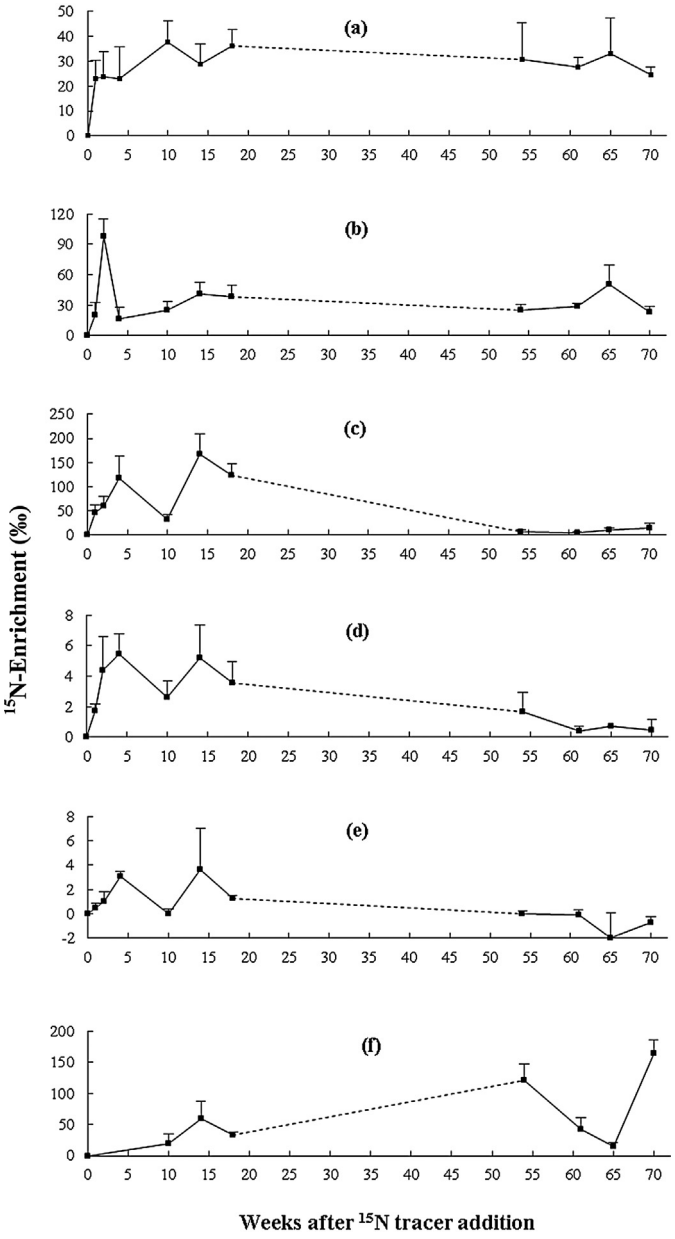

Fig. 1. ^{15}N enrichment (‰) of the heath compartments analyzed for 10 sampling occasions (7 for microbial biomass) following ^{15}N tracer addition (weeks after ^{15}N tracer addition). a, new shoots; b, old shoots; c, O-horizon; d, A-horizon; e, B-horizon; f, soil microbial biomass. Broken lines between weeks 18 to 54 represent the absence of sampling in this period.

3.3. ^{15}N tracer recovery

Table 2 shows the results of ^{15}N tracer recovery. Total ^{15}N tracer recovery (overall compartments) was 71.54% in the first year, and 5.09% in the second year of the experiment. Tracer recovery in *Calluna* shoots was low (always below 1.9% in both years). Recovery tended to be higher in old shoots, but differences between old and new shoots were significant only in the second year ($F_{(1,6)} = 6.632$, $p < 0.05$). Recovery rates in shoots decreased in the second year. ^{15}N tracer recovery was highest in the O-horizon (for both years), but recovery significantly decreased in the second year ($F_{(1,3)} = 50.28$, $p < 0.05$). A- and B-horizons had a lower ^{15}N recovery, with means

Table 1
^{15}N natural abundances of the *Calluna* biomass, soil and soil microbial biomass in the control subplots.

Date of sampling	Weeks after ^{15}N addition	New shoots	Old shoots	O-horizon	A-horizon	B-horizon	Microbial biomass
2011							
July/07	1	−2.13 (0.70)	−1.78 (0.56)	0.97 (0.68)	5.00 (0.30)	7.09 (0.31)	ND
July/14	2	−1.29 (0.58)	−1.82 (0.65)	0.52 (0.72)	4.72 (0.79)	7.20 (0.53)	ND
July/26	4	2.74 (3.54)	1.19 (2.80)	0.54 (0.57)	4.28 (0.60)	6.53 (0.05)	ND
Sept./05	10	−1.35 (0.82)	−1.52 (0.75)	1.35 (0.56)	4.55 (0.78)	7.63 (0.64)	−3.68 (0.80)
Oct./03	14	−1.36 (0.66)	−2.26 (0.62)	−0.45 (0.58)	4.14 (1.33)	7.68 (0.89)	−3.43 (3.94)
Nov./04	18	−1.88 (0.80)	−1.74 (0.61)	0.16 (0.40)	4.34 (0.82)	7.28 (0.18)	3.15 (2.37)
2012							
July/11	54	−1.62 (0.77)	−1.63 (0.63)	4.26 (0.62)	6.56 (0.08)	8.23 (0.36)	−3.81 (5.80)
Aug./28	61	−1.58 (0.63)	−2.06 (0.68)	3.63 (1.06)	6.96 (0.33)	8.19 (0.46)	4.37 (3.34)
Sept./28	65	−1.51 (0.85)	−2.86 (0.50)	4.41 (1.25)	6.60 (0.46)	8.16 (0.04)	10.85 (6.34)
Nov./04	70	−1.99 (0.81)	−1.63 (0.85)	4.31 (0.91)	7.49 (0.46)	8.86 (0.28)	−2.95 (7.56)

Data are means of $\delta^{15}N$ (‰) with SE in parentheses. Negative values indicate depletion and positive values indicate an enrichment of $^{15}N/^{14}N$ ratio in the sample compared with atmospheric N_2 ($\delta^{15}N = 0$‰). Samples of microbial biomass were not collected on the first three sampling occasions (ND).

Table 2
^{15}N tracer recovery of the heath compartments analyzed for two late growing season dates (November 2011 and November 2012).

Compartment		November 2011				November 2012			
		$^{15}N_{rec}$ (mg N m^{-2})		% $^{15}N_{rec}$		$^{15}N_{rec}$ (mg N m^{-2})		% $^{15}N_{rec}$	
New shoots		0.21	(0.04)	0.54	(0.10)	0.12	(0.01)	0.32	(0.02)
Old shoots		0.51	(0.14)	1.31	(0.37)	0.28	(0.06)	0.73	(0.16)
O-horizon		18.03	(3.11)	46.58	(8.04)	1.04	(0.79)	2.69*	(2.04)
A-horizon		5.05	(1.95)	13.04	(5.03)	0.52	(0.84)	1.33	(2.16)
B-horizon		3.89	(1.01)	10.06	(2.61)	0.00	(0.00)	0.00	(0.00)
Soil microbial biomass		0.08	(0.01)	0.22	(0.04)	0.59	(0.13)	1.52*	(0.33)
Leaching losses	$^{15}NO_3^-$	0.003		0.007		0.003		0.009	
	$^{15}NH_4^+$	0.000		0.001		0.001		0.003	
Total recovery (%)				**71.54**				**5.09**	

Data are means with SE in parentheses. ^{15}N tracer recovery is expressed as total mass of ^{15}N tracer recovered ($^{15}N_{rec}$) and as a percentage of total ^{15}N tracer masses (%$^{15}N_{rec}$). Soil microbial biomass was included within O-horizon for the calculation of ^{15}N total recovery.
Leaching losses are given as sum of ^{15}N leaching losses since ^{15}N tracer addition.
* Significant differences in ^{15}N tracer recovery between November 2011 and November 2012 ($p < 0.05$).

of 13.04% and 10.06% in the first year, respectively. Tracer recovery in these horizons tended to be lower in the second year. ^{15}N recovery decreased with soil depth in both years, but differences were significant only in the first year ($F_{(2,9)} = 12.758$, $p < 0.05$). As regards soil microbial biomass, only 0.5% of ^{15}N tracer in the O-horizon was located in microbial biomass in the first year, but this proportion increased to 56.5% in the second year. This corresponded to an increase of ^{15}N recovery in the soil microbial biomass from 0.22% in the first year to 1.52% in the second year ($F_{(1,3)} = 12.746$, $p < 0.05$). Besides, no clear relation was observed between N content of O-horizon or precipitation and ^{15}N tracer recovery in soil microbial biomass (Table 3). ^{15}N tracer recovery in leachate was very low in both years of experiment, with higher values for $^{15}N{-}NO_3^-$ as compared to $^{15}N{-}NH_4^+$.

3.4. ^{15}N leaching losses

^{15}N enrichment in leachate showed no significant shifts over time. Enrichment was higher in $N{-}NO_3^-$ than in $N{-}NH_4^+$ during the first year ($N{-}NO_3^- = 60.21$‰; $N{-}NH_4^+ = 30.00$‰), and the second year ($N{-}NO_3^- = 39.78$‰; $N{-}NH_4^+ = 26.99$‰). ^{15}N losses by leaching were low in both years, accounting for 0.012% of total ^{15}N tracer added (Fig. 2). Thus, ^{15}N losses via leaching were much lower as compared to the amount of ^{15}N tracer retained by *Calluna*

Table 3
^{15}N tracer recovery and N content of O-horizon and soil microbial biomass, and precipitation in all sampling dates.

Date of Sampling	Weeks after ^{15}N addition	Precipitation (mm)	O-horizon		Soil microbial biomass	
			%$^{15}N_{rec}$	g N m^{-2}	%$^{15}N_{rec}$	g N m^{-2}
2011						
July/07	1	2.4	12.47 (4.75)	26.76 (4.64)	ND	ND
July/14	2	65.1	17.57 (5.71)	30.28 (2.83)	ND	ND
July/26	4	16.0	43.80 (17.83)	36.07 (3.87)	ND	ND
Sept./05	10	38.8	8.97 (3.19)	25.88 (3.25)	0.08 (0.10)	0.65 (0.11)
Oct./03	14	7.0	66.07 (17.45)	40.51 (1.42)	0.47 (0.23)	0.77 (0.09)
Nov./04	18	144.0	46.58 (8.04)	39.59 (1.08)	0.22 (0.04)	0.66 (0.07)
2012						
July/11	54	47.7	1.61 (1.07)	25.54 (1.78)	0.74 (0.31)	0.58 (0.13)
Aug./28	61	21.7	0.95 (0.75)	21.62 (3.26)	0.46 (0.26)	1.08 (0.19)
Sept./28	65	22.2	2.33 (0.58)	24.16 (0.74)	0.15 (0.06)	1.06 (0.02)
Nov./04	70	157.7	2.69 (2.04)	16.30 (2.91)	1.52 (0.34)	0.96 (0.18)

Data are means of ^{15}N tracer recovery (%) and means of N content (g N m^{-2}) with SE in parentheses. Precipitation data (mm) are means of rainfall since last sampling occasion, except for July/07 (2011) and July/11 (2012), which are in last week and in last month respectively. Samples of soil microbial biomass were not collected on the first three sampling occasions (ND). ^{15}N tracer recovery and N content of soil microbial biomass are also included within O-horizon data as a part of this compartment.

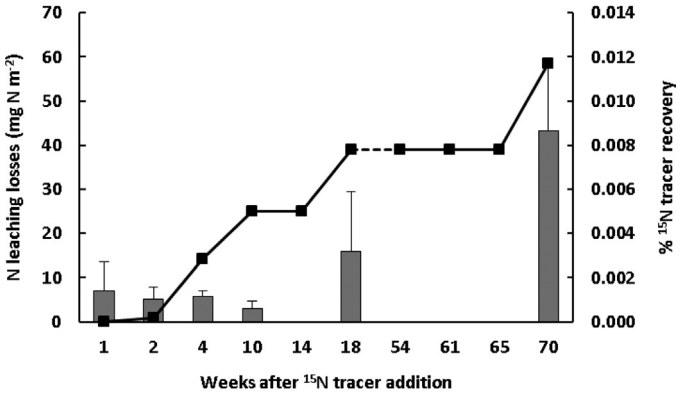

Fig. 2. ^{15}N leaching losses expressed as a cumulative curve of ^{15}N recovery (%) compared to N leaching losses (expressed as filled bars; in mg N m^{-2}). Broken line between weeks 18 to 54 represents the absence of sampling in this period. N leaching losses and ^{15}N tracer recovery in the first two weeks are only NO_3^- data.

shoots, soil horizons or soil microbial biomass (Table 2). ^{15}N losses by leaching were detectable in the fourth week after tracer addition (Fig. 2). No leaching losses appeared in weeks 14, 54, 61 and 65 (Fig. 2), which coincided with dry periods in summer (Table 3). We found a clear relationship between the amount of N leached and ^{15}N recovery in the leachate (Fig. 2). ^{15}N tracer losses peaked in weeks 18 and 70 (Nov. 2011 and Nov. 2012), coinciding with high rainfall periods at the end of the growing season in the Cantabrian Mountains (Table 3).

4. Discussion

4.1. ^{15}N partitioning and recovery

The soil (and the O-horizon in particular) represented the compartment with the highest capacity of N retention in Cantabrian Mountains heathlands, as occurs in other heathlands in the UK (Curtis et al., 2005). Approximately 47% of the ^{15}N tracer added was recovered in the organic horizon. This coincided with results found by other authors (Tietema et al., 1998; Nadelhoffer et al., 2004; Perakis et al., 2005; Sheng et al., 2014) for different type of forest ecosystems. However, in studies conducted in heathlands (Germany) with a dense bryophyte cover, the moss layer was shown to act as the most important N immobilizer (Friedrich et al., 2011a). The high N storage capacity of the organic horizons can be explained by several factors. First, organic layers have high organic matter contents and thus are characterized by high cation exchange capacities, which in turn support the sorption of cations such as NH_4^+ (Turner and Henry, 2009; Wang and Zhu, 2012). Second, N can be retained due to biotic immobilization (i.e. the microbial biomass), and microbial community has shown a high capacity to immediately capture the N deposited in the ecosystem (Kristensen and McCarty, 1999; Southon et al., 2012). In our study we found only high values of ^{15}N recovered in the microbial biomass compared to ^{15}N recovered in the O-horizon in the second year (i.e. 56.7% in 2012), while in other experiments about 75% of ^{15}N present in the organic horizons was already fixed by microbes in the first few weeks after application (Green, 2005 (cited in Green et al., 2013); Tye et al., 2005). The delayed recovery rates found in our experiment could be explained by the climatic characteristics of the Cantabrian heathlands, i.e. periods of summer drought that regularly appear throughout the growing season (Loidi et al., 2010). These drought events negatively affect the N uptake and thus N immobilization by soil microbes (Nielsen et al., 2009; Green et al., 2013). We therefore hypothesize that a great amount of ^{15}N tracer was retained in inorganic form in the soil during the first year (Sheng et al., 2014), which then gradually changed into organic forms (Tye et al., 2005) due to the uptake by soil microbes (Dunn et al., 2006). Mineral horizons (such as the A- and B-horizons) have low N retention capacities compared to the O-horizon. However, in Podsol soils (typical in *Calluna*-heathlands) there is an important nitrogen accumulation in mineral horizons (Pilkington et al., 2005b; Friedrich et al., 2011a). As a general pattern, the rate of ^{15}N tracer recovered from the soil horizons of the studied heathlands decreased between the two years, indicating that there were ^{15}N tracer losses over time.

The low recovery of ^{15}N tracer in aboveground biomass of *Calluna*, both new and old shoots, is indicative of a slow process of N replacing, characteristic of N unsaturated ecosystems (Högberg, 1997). *Calluna* shoots showed similar ^{15}N enrichment in both old and new shoots, and this could be due to the nutrient dynamics of mature *Calluna* (>40 years) present in our study area. Large differences in ^{15}N tracer recovery between *Calluna* aboveground biomass and soil horizons were also found by Power et al. (1998), suggesting that *Calluna* shoots represent a small sink for N (likely due to high C:N ratios and thus comparatively low N demands), even under N limited growths. Therefore, most of the N entering the ecosystem is retained in the O-horizon, and *Calluna* aboveground biomass incorporated only a very small fraction. In addition, N uptake in plant biomass could also be hampered by the low P availability (Britton et al., 2008; Friedrich et al., 2011b; Jones and Power, 2012) that was found in these heathlands (Villalón, 2014). Another possible reason may be due to the ability of *Calluna* plants to incorporate N as NH_3 in gaseous form through the aboveground biomass (Jones et al., 2008), reducing the N acquisition from the soil, and therefore the ^{15}N tracer acquisition. Although Schimel and Bennett (2004) pointed out that *C. vulgaris* competes worse for N than soil microorganisms, the poor ^{15}N tracer recovery obtained in soil microbial biomass of the Cantabrian heathlands showed that this competition for nutrients is not the main explanation of low N incorporation in *Calluna* biomass. This argument is reinforced by the fact that the soil microbes prefer N in an organic form (Dunn et al., 2006).

4.2. ^{15}N leaching losses

Low N losses through leaching confirmed our hypothesis that the heathlands analyzed are still N limited, despite currently prevailing rates of atmospheric N inputs. There was a delay of two weeks after ^{15}N application until the tracer could be detected in the leachate. This roughly indicates the time the tracer needed to pass the soil matrix, likely due to downward transport with seepage water. A delay phase of two weeks was also observed by Friedrich et al. (2011a) in heathlands of NW Germany. The highest losses of ^{15}N by leaching occurred during high rates of precipitation, indicating ^{15}N losses due to greater amounts of seepage water after rainfall events (Power et al., 2006). N losses with seepage water are mediated by soil characteristics and the composition and structure of heathland vegetation (Herrmann et al., 2005). Soils with high organic matter content, typical of our study area (Marcos et al., 2003), are associated with very low N losses by leaching (Evans et al., 2006), because organic matter increases the soils' N retention capacity (Wang and Zhu, 2012). N leaching in the studied heathlands mainly occurred in the form of NO_3^-, with NO_3^- leaching losses three times higher than corresponding NH_4^+ losses (Herrmann et al., 2005). This finding may be due to (i) the better absorption of NH_4^+ to the soil cation exchange sites (Stevens et al., 2011), (ii) the faster uptake of NH_4^+ by plants (Bloom et al., 1992).

4.3. Not quantified losses

The ^{15}N tracer not recovered in this experiment (28.46%) could be explained because it has been accumulated in compartments

that were not analyzed in this study: i.e. woody biomass from *Calluna* or other species which showed very low cover values. Moreover, some N losses can be attributed to the transport of nutrients and other chemical compounds (Achatz and Rillig, 2014) by hyphae, although these losses were not quantified. We did not expect important losses by volatilization or denitrification in a short time. We think that the highest proportion of not recovered ^{15}N during the second year is related to the denitrification process that usually occurs in water-saturated soils (Wolf and Russow, 2000). Mathieu et al. (2006) found that denitrification contributed around 85–90% to N_2O flux in saturated conditions. In Cantabrian heathlands during the winter and early spring the soils are completely water-saturated, so a high level of denitrification is to be expected.

5. Conclusions

This study uses a ^{15}N tracer to determine the allocation patterns of airborne nitrogen in Cantabrian heathlands. The results demonstrated that these Cantabrian heathlands could retain about 72% of N applied in a short time and showed low N losses via leaching. These findings support our hypothesis that Cantabrian heathlands are still not N saturated. Moreover, it was observed that the soil organic horizon was mainly responsible for rapid immobilization of 47% of the N applied. Losses of ^{15}N during the second year were very significant (around 95%), which could be due to the denitrification process (not quantified) during the winter and spring. The results suggest that after initial N retention, the ecosystem could transfer a considerable N flux to the atmosphere. In this way, the ecosystem can remain in a non-N saturated state (despite current atmospheric inputs). For this reason, further studies would be necessary to evaluate nitrogen fluxes in these ecosystems and their contribution to climate change.

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