Effects of atmospheric nitrogen deposition on the functioning, structure, and composition of *Calluna vulgaris* (L.) Hull heathlands in the Cantabrian Mountains (NW Spain): A critical load approach.

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Abstract

Calluna-vulgaris-heathlands located at the southern-most distribution area (Cantabrian Mountains, NW Spain) are currently threatened by global change drivers such as atmospheric nitrogen (N) depositions that affect their functioning, structure and plant species composition. The main aim of the present Doctoral Thesis was to evaluate the effects of different atmospheric N loads in Cantabrian Mountains heathlands at two different life-cycle stages (young- and mature-phase).

Field measured bulk inorganic N deposition rates in the Cantabrian Mountains ranged between 2.8 kg N ha^{-1} yr^{-1} and 4.6 kg N ha^{-1} yr^{-1}, with higher N deposition rates during the wet period. The prevailing N chemical form in atmospheric depositions is from oxidized compounds (NO_Y). The distribution and fate of these airborne N inputs in the *Calluna*-heathland were traced with ^{15}N isotope. These heathlands were able to retain ~72% of total N inputs in the short-term (< 1 year). The soil organic horizon immobilized ~47% of the N inputs. During the second year, ~95% of N inputs was likely lost by denitrification fluxes during the winter-spring period. Leaching losses were negligible over two years, suggesting that these heathlands were not N saturated.

Furthermore, we aimed to evaluate the effects of different levels of experimentally simulated N deposition on the functioning, structure and composition of Cantabrian *Calluna*-heathlands. The increase in atmospheric N loads resulted in a subsequent increase in soil ammonium (NH_4^+) with long-term N inputs (10 years); although soil nitrate (NO_3^-), total N, organic carbon (C), and available phosphorus (P) pools remained unchanged. Besides, *Calluna* shoot N and P contents and litter N content also increased, which was accordingly reflected in higher N:P ratios. The greatest P demand by *Calluna*

biomass from P-poor soils was supplied by the increase in acid phosphatase enzymatic activity to mineralize organic P, and by the extent of *Calluna* roots colonized by mycorrhizal fungi to support the higher P uptake. There were no changes in soil microbial biomass C and N contents in response to higher N availability, as well as in urease (N cycle) and β-glucosidase (C cycle) enzymatic activities associated to microbial nutrient demand. The lack of response of several soil properties and microbial nutrient contents could be due to extreme climatic conditions and short period of physiological activity in these montane areas, indicating that montane heathlands are highly resistant to increased N loads. Furthermore, plant community composition and plant species richness remained unchanged in response to enhanced N inputs, likely due to the resistant and resilient mechanisms of heathland dominant vegetation. However, at life-form level, percentage of cover of N-tolerant graminoids and forbs species increased with higher N availability, while the cover of bryophytes and lichens declined. There was a stimulation of *Calluna vulgaris* vital rates (shoot growth and flowering) in response to short-term N inputs, but the first symptoms of N saturation were observed under long-term N inputs (10 years) through a declined trend in these vital rates. The heathland life-cycle stage conditioned the response to enhanced N loads through stronger N-related effects in young stands compared to mature ones. In Cantabrian heathlands, the N critical load was set in the range of 10-20 kg N ha^{-1} yr^{-1} for both young and mature stands, based mainly on changes in *Calluna* flowering and shoot N content at 14.6 kg N ha^{-1} yr^{-1}.

Resumen

La conservación de los brezales de *Calluna vulgaris* localizados en su límite más meridional de distribución (Cordillera Cantábrica, NO España) está actualmente amenazada por vectores de cambio global como la deposición atmosférica de nitrógeno (N), la cual constituye una amenaza para la calidad del ecosistema debido a cambios en su funcionamiento, estructura vegetal, y composición de especies. En esta Tesis Doctoral se pretende evaluar el efecto de diferentes cargas de deposición de N en la estructura y funcionamiento de los brezales de *Calluna vulgaris* de la Cordillera Cantábrica bajo la influencia de la fase de desarrollo de la comunidad vegetal (ej.: fase joven y fase madura).

En primer lugar, se midieron en campo las tasas de deposición total de N inorgánico, las cuales variaron desde 2,8 kg N / ha año en La Majúa hasta 4,6 kg N / ha año en San Isidro, siendo más elevadas durante el periodo de mayor precipitación. Las formas químicas oxidadas de N (NO_Y) fueron predominantes en las deposiciones atmosféricas. Posteriormente, la distribución y el destino de estas deposiciones en el ecosistema de brezal de *Calluna vulgaris* fueron seguidos mediante el uso del trazador ^{15}N. Estos ecosistemas fueron capaces de retener alrededor del 72% de las entradas de N a corto plazo (< 1 año), siendo el horizonte orgánico del suelo el sumidero del 47% de las entradas de N en el ecosistema. Durante el segundo año, el 95% de N del ecosistema se perdió a través de procesos de desnitrificación durante el invierno y la primavera. Las pérdidas de N por lixiviación fueron inapreciables durante dos años de estudio, sugiriendo que el ecosistema de brezal de *Calluna vulgaris* no estaba saturado en N.

A continuación se evaluaron los efectos de diferentes cargas de N en el funcionamiento, estructura, y composición de estos ecosistemas. El incremento en las cargas de N causó un aumento de la concentración de amonio (NH_4^+) del suelo bajo elevadas (56 kg N / ha año) cargas crónicas (10 años), mientras que las concentraciones de nitrato (NO_3^-), N total, carbono (C) orgánico, y fósforo (P) asimilable no fueron alteradas. El contenido de N y P en los brotes de *Calluna* y el contenido de N en la hojarasca también se incrementaron como respuesta a mayores cargas de N, lo que se reflejó en una elevada relación N:P. La mayor demanda de P por la biomasa de *Calluna* fue suministrada mediante el incremento de la actividad enzimática fosfatasa ácida necesaria para la mineralización de P orgánico, y mediante una mayor colonización de raíces de *Calluna* por hongos micorrícicos con el objeto de facilitar la mayor absorción de P. Por el contrario, no hubo cambios en el contenido de C y N de la biomasa microbiana del suelo, ni en las actividades enzimáticas ureasa (ciclo del N) y β-glucosidasa (ciclo del C) en respuesta al aumento de la disponibilidad de N. La ausencia de efectos en varias propiedades del suelo y en el contenido de nutrientes microbianos podría ser debido a las condiciones climáticas extremas y al corto periodo de actividad vegetativa de estos sistemas de montaña. Por tanto, los resultados obtenidos señalan que los brezales de *Calluna vulgaris* de alta montaña son resistentes al aumento de una mayor disponibilidad de N. En cuanto a la comunidad vegetal, la composición y la riqueza de especies tampoco fueron modificadas, lo cual probablemente se relacionó con los mecanismos de resistencia y resiliencia desarrollados por la vegetación dominante de estas comunidades. Sin embargo, a nivel de formas de vida, hubo un incremento en la cobertura de graminoides (tolerantes al N) y herbáceas perennes, mientras que la cobertura de briófitos y líquenes se redujo. Los rasgos vitales de la especie dominante

Calluna vulgaris (crecimiento de brotes y floración) se vieron positivamente afectados por un aumento de las cargas de N a corto plazo, pero en cambio, se observaron los primeros síntomas de saturación bajo cargas crónicas (10 años) mediante una disminución del crecimiento de brotes y de la floración. La fase del ciclo de vida del brezal de *Calluna vulgaris* presentó una gran influencia en la respuesta del ecosistema al incremento de las cargas de N, ya que los brezales jóvenes mostraron efectos más intensos relacionados con estas deposiciones de N que los brezales maduros.

Finalmente, se determinó la carga crítica para la deposición de N en los brezales de *Calluna vulgaris* de alta montaña, teniendo en cuenta su fase de desarrollo (fase joven o de crecimiento, y fase madura). La carga crítica basada en cambios significativos de la floración de *Calluna* y el contenido de N de los brotes para formaciones jóvenes y cambios en la floración de *Calluna* en formaciones maduras fue de 14,6 kg N / ha año. Esta carga se encuentra dentro del actual rango de cargas críticas para brezales secos (10-20 kg N ha / año).

Introduction

Heathlands: trends and current status

Heathlands are considered as traditional semi-natural habitats characterized by the dominance of perennial sclerophyllous heather species, which are developed in oceanic climates with high rainfall and low annual temperature oscillation (Loidi et al., 2010). Traditional heathland habitats, and particularly those dominated by the dwarf-shrub *Calluna vulgaris* (L.) Hull, have been widely recognized as historical cultural landscapes at European level (Webb, 1998; Fowler et al., 2003; Fagúndez, 2013; Morán-Ordóñez et al., 2013). These *Calluna vulgaris* heathlands are distributed along the Atlantic coast in Western Europe, as well as in lowland areas of Central Europe (Fig. 1).

Figure 1. Distribution of *Calluna vulgaris* dominated heathlands in Europe. Source: Kvamme et al. (2004).

In general, *Calluna*-heathlands have been traditionally managed to provide pastures for livestock grazing through practices such as prescribed burning, cutting, and mowing (Barker et al., 2004; Newton et al., 2009; Jones et al., 2017) (Fig. 2). However, land abandonment during the twentieth century for socio-economic reasons and lack of traditional activities associated to heathland maintenance have resulted in a drastic decline and fragmentation of these communities in recent decades (Fagúndez et al., 2013; Morán-Ordóñez et al., 2013), with more than 90% of heathland areas having disappeared (de Blust, 2007). These heathlands were replaced by shrublands or woodlands through secondary succession (Moen et al., 2006; Morán-Ordóñez et al., 2011), since *Calluna vulgaris* plants would have reached the degenerate life-cycle stage (Fig. 2), or have been changed for other anthropogenic-related land uses such as arable fields or urban areas (Morán-Ordóñez et al., 2013). For this reason, in order to preserve heathlands, they have been considered as priority conservation areas by the European Habitats Directive (Natura 2000 network; Annex I Habitats Directive, 92/43/ECC).

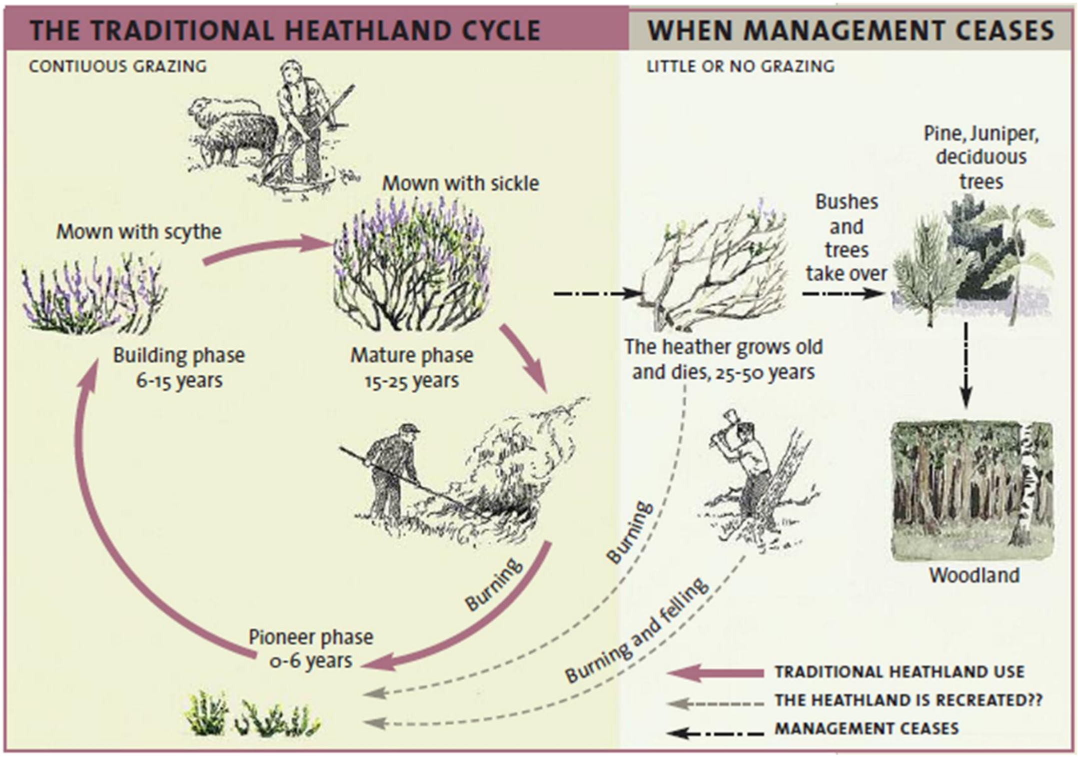

Figure 2. Scheme of traditional management cycle in heathland systems. Source: Kvamme et al. (2004).

Particularly, *Calluna*-heathlands in the Iberian Peninsula are mainly located in the Cantabrian Mountains (North-Western Spain), and represent the southern-most distribution area of this ecosystem type in Europe (Fig. 1; Kvamme et al., 2004). These mountainous heathlands are made up of small dispersed patches (Calvo et al., 2002a), although they are recognized as a diversity hotspot due to the great number of species (plants, birds, and invertebrates) and ecosystems they host (de Bello et al., 2010; Morán-Ordóñez et al., 2011). Furthermore, heathlands located in NW Spain have been reported as the highest floristic richness areas of European heathlands (Loidi et al., 2010), since they are located in the ecotone between the Atlantic/Eurosiberian and Mediterranean biogeographical regions. Traditionally, the Cantabrian Mountains heathlands have been used to provide a wide range of ecosystem services such as pastures for livestock, biomass fuel, and food products, among others. Nowadays, however, socio-economic changes have had a detrimental effect on the provision of these ecosystem services (Morán-Ordóñez et al., 2013). Historically, these Cantabrian heathlands have been subjected to frequent perturbations associated to management practices including burning, cutting, and grazing by transhumant sheep (Morán-Ordónez et al., 2013), as well as to environmental stress (drought periods and increasing atmospheric N depositions) (Marcos et al., 2003, 2009; Calvo et al., 2005a, 2007; Cuesta et al., 2008; Meyer-Grünefeldt et al., 2016; Taboada et al., 2016). The singular environmental features and historical management practices in Cantabrian Mountains heathlands have conferred different behaviour patterns in relation to environmental perturbations such as atmospheric N inputs compared to lowland heathlands in central and northern Europe (Meyer-Grünefeldt et al., 2016).

Current loads and fate of atmospheric nitrogen depositions

The increase in anthropogenic reactive nitrogen creation (Nr; defined as all N compounds except unreactive N_2) since the 1950s by growing food and energy production (Fig. 3; Galloway et al., 2014) has resulted in a significant alteration in the N cycle worldwide (Mosier et al., 2001; Galloway et al., 2004). For the following decades, estimations have predicted a progressive increase in Nr at global scale, which will be incorporated into global N cycling (Galloway et al., 2004).

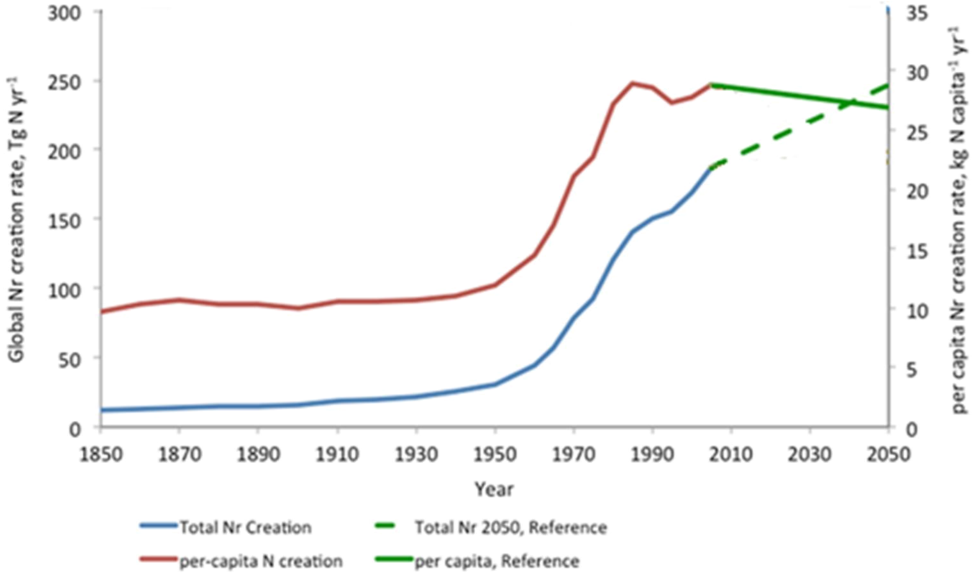

Figure 3. Temporal trends in global anthropogenic Nr creation on a total and per-capita basis. Source: Galloway et al. (2014).

In Spain, the projections of N emissions for the period 2015-2020 have shown different trends depending on the N chemical form: a decrease of 16% for NO_X emissions (N-oxidized form) and a slight increase of 2% for NH_3 emissions (N-reduced form) (EEA, 2014), according to European Union legislation requirements. Since the 2000s, the successful implementation of air quality policies to control nitrogenous pollutants have led to a reduction in both NO_X and NH_3 emissions (Cuevas et al., 2014; Vedrenne et al., 2015; Aguillaume et al., 2016), which are related to the observed decreases in statewide atmospheric NO_X and NH_3 depositions in recent years (Izquierdo and Àvila, 2012; Vedrenne et al., 2015; Aguillaume et al., 2016). Particularly, the application of air quality policies in North-Western Spain have led to a sharp decline in atmospheric NO_X concentration of about 52% from 1996 to 2012 (Cuevas et al., 2014), which would be reflected in a similar reduction in NO_X depositions in the surrounding area. In addition, progressive land abandonment in the Cantabrian Mountains (NW Spain) since the 1950s (Morán-Ordóñez et al., 2013) could have contributed to a reduction in NH_X depositions (gaseous NH_3 and particulate NH_4^+) associated to NH_3 emissions from livestock and agricultural activities. The accurate quantification of atmospheric N depositions is increasingly important to understanding their ecological impacts in terrestrial ecosystems (Zhang et al., 2008; Boutin et al., 2015), especially in those mountainous systems which are recognized as highly N-sensitive (Britton et al., 2008; Britton and Fisher, 2008). However, only modeled atmospheric N deposition data are currently available for the Cantabrian Mountains in recent years (7.5-15 kg N ha^{-1} yr^{-1}; García-Gómez et al., 2014), with a lack of field measured data on N depositions in these areas. Besides, the prevailing chemical form in airborne N depositions (oxidized or reduced) has been reported as a relevant factor that influences the dynamic of N cycling and the ecological impacts (Boutin et al., 2015; van den Berg et al., 2016).

In general, the distribution and fate of current atmospheric N depositions are relatively unknown in many terrestrial ecosystems (Galloway et al., 2004). In the mid-90s, only the fate of 35% of atmospheric N inputs was well known, with uncertainties about the fate of the remaining 65% N inputs (Galloway et al., 2008). Understanding the allocation patterns of N inputs and storage within the different ecosystem compartments (i.e., plant biomass, soil horizons, soil microbes, and fluxes such as leaching losses) could be of great importance in evaluating the potential impacts of current atmospheric N depositions (Friedrich et al., 2011a). Besides, the quantification of N flux rates such as leaching losses will provide valuable information about the ecosystem N-saturation status (Friedrich et al., 2011a). Despite the recent decline in atmospheric N depositions, there are no studies to date about the distribution and fate of current airborne N inputs within the heathland ecosystems in the Cantabrian Mountains, which are essential to design the appropriate management strategies to preserve these ecosystems.

Critical loads for atmospheric nitrogen depositions

Calluna-heathlands are adapted to low levels of N availability (De Graaf et al., 2009), and therefore, increasing N inputs could result in negative effects on ecosystem health (Gao et al., 2014). The critical load concept has been established as a useful tool to assess the potential impacts of atmospheric N depositions on ecosystem components (Pardo et al., 2011; Roth et al., 2017). It has been defined as the critical threshold for N depositions below which no harmful effects are expected on ecosystem components or their services (Nilsson and Grennfelt, 1988). Currently, the critical load for dry heathlands is established at 10-20 kg N ha^{-1} yr^{-1} (Bobbink and Hettelingh, 2011). However, the *Calluna*-heathland life-cycle stage should be taken into account in the estimation of N critical loads, since heathland stand age is able to respond differently to N inputs (Jones and Power, 2015; Meyer-Grünefeldt et al., 2015).

The estimation of N critical loads for dry heathlands dominated by *Calluna vulgaris* is based on observed changes in their structure, composition, and functioning through field manipulative experiments with increasing N loads. Bobbink and Hettelingh (2011) proposed four indicators for exceedance of the N critical threshold in dry heathlands: i) transition heather to grass, ii) decline in lichens, iii) changes in plant biochemistry, and iv) increased sensitivity to abiotic stress.

Regarding plant species composition, there will be a wide loss of oligotrophic species with increasing N availability, since they will be less competitive than the nitrophilous ones (Maskell et al., 2010; Stevens et al., 2011; Roth et al., 2013). Besides, higher N availability is related to increased susceptibility to environmental stress factors such as frost injuries, drought damage, and *Lochmaea suturalis* heather beetle defoliation (Sheppard et al., 2008; Taboada et al., 2016; van Voorn et al., 2016). In addition, cryptogam species such as bryophytes and lichens have shown that they are very sensitive to N inputs (Edmondson et al., 2013; Bähring et al., 2017), being replaced by N-tolerant grasses (Remke et al., 2009; Phoenix et al., 2012; Southon et al., 2013).

Regarding ecosystem functioning, a complex network of ecosystem impacts in response to enhanced N inputs has been identified (Fig. 4; Bobbink et al., 2010). In a summarized form, higher N availability will primarily result in increased plant and litter production; followed by nutrient imbalances towards P limitation for plants and soil microbes, and greater plant and litter N contents (Bobbink et al., 2010). Higher N mineralization rates and soil acidification will occur as the last symptoms of N inputs. Furthermore, changes in the nutrient content of soil microbial biomass and soil enzymatic activity rates are also expected in response to increased N inputs (Johnson et al., 1998; Pilkington et al., 2005; Ramírez et al., 2012).

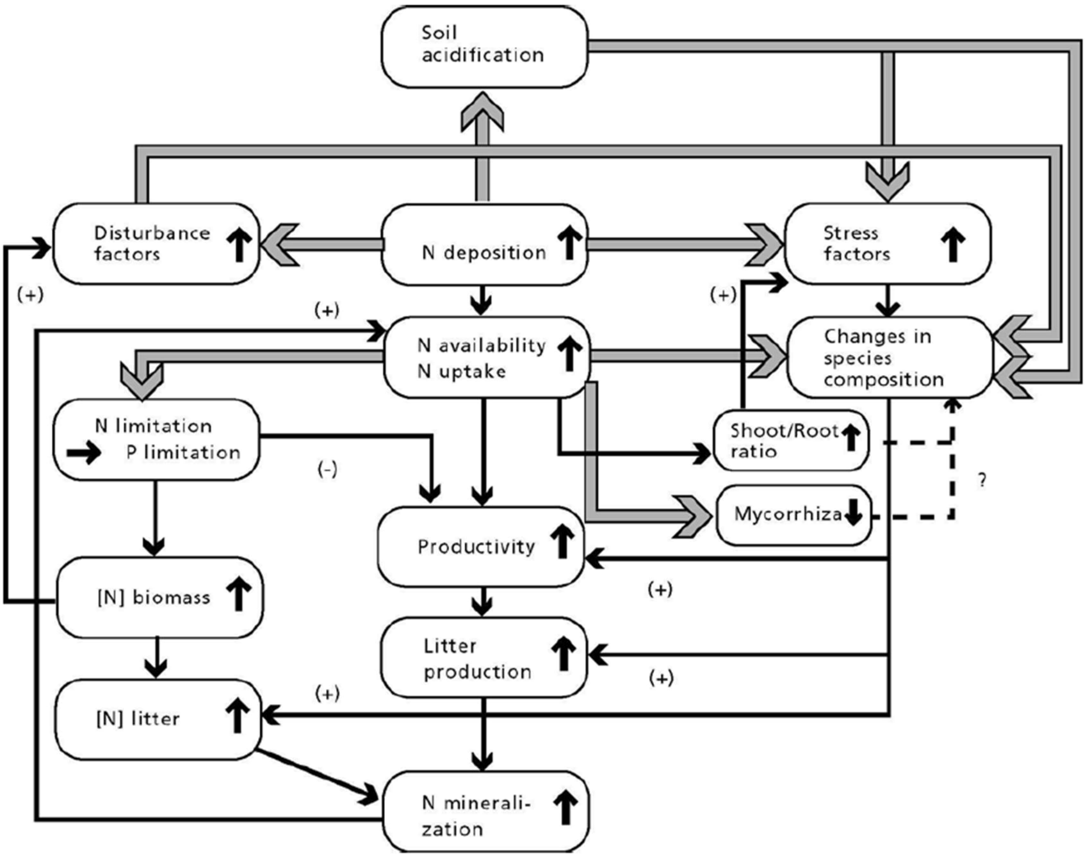

Figure 4. Scheme of the main impacts of enhanced N deposition on ecosystems. ↑ indicates increase; ↓ decrease; small arrow: effect will occur in the short term (< 5 years); wide arrow: indicates long-term effect. (+): positive feedback; (-) negative feedback. Source: Bobbink et al. (2010).

Justification of the study

To date, the effects of increasing atmospheric N depositions in heathlands have been widely studied in lowland and upland *Calluna vulgaris* heathlands in central and northern Europe through experimental N manipulative studies (Power et al., 1998; Britton et al., 2008; von Oheimb et al., 2010; Friedrich et al., 2011b; Southon et al., 2012; Meyer-Grünefeldt et al., 2015; Bähring et al., 2017; among others) and field-scale surveys (Rowe et al., 2008; Jones and Power, 2012; Southon et al., 2013; among others) since these heathlands are considered of priority conservation at a European level (European dry heaths; Habitats Directive 92/43/EEC). However, it is expected that *Calluna vulgaris* heathlands located in their southern-most distribution area (Cantabrian Mountains, NW Spain) are able to display different behaviour in relation to airborne N inputs compared to lowland heaths in central and northern Europe (Meyer-Grünefeldt et al., 2016). The Heathland Ecology group of the University of León has carried out different studies in the Cantabrian Mountains heathlands since 1987, focusing on (1) characterization of their vegetation, soil, and edaphofauna; (2) understanding the effects of landscape changes on species distribution; and (3) the analysis of the effects of perturbations, such as burning and cutting, on the structure and functioning of these ecosystems (Valbuena et al., 2000; Calvo et al., 2002a, 2002b, 2005b, 2012; Cuesta et al., 2006; Marcos et al., 2009; Morán-Ordóñez et al., 2011, 2013). Since 1998, this research group has performed several studies on the alteration of heathland structure and functioning under the framework of increased N deposition (Marcos et al., 2003; Calvo et al., 2005a, 2007; Cuesta et al., 2008; Taboada et al., 2016). The present Doctoral Thesis arises from the need to know the critical loads for atmospheric N depositions in these N-sensitive *Calluna* heathlands in the Cantabrian Mountains, based on harmful effects of airborne N inputs on the vegetation structure, plant species composition, and ecosystem functioning. Another key point is to know the current rates of N depositions, as well as the distribution and fate of these N inputs within the heathland ecosystem compartments in order to develop suitable management policies to protect them against environmental perturbations such as enhanced N inputs.

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Objectives

The main objective of this Doctoral Thesis is to evaluate the effects of different levels of N loads on the vegetation structure, plant species composition, and ecosystem functioning of montane *Calluna vulgaris* heathlands, mediated by the time-scale of N inputs and the age of heathland vegetation (i.e., young- or building-phase and mature-phase) resulting from management practices like prescribed burning.

This main objective was divided into the following specific aims:

1. To assess the field-measured atmospheric bulk inorganic N depositions in Cantabrian heathlands and determine temporal patterns of N deposition rates, as well as to quantify inorganic N chemical forms in atmospheric depositions (oxidized/reduced). *Chapter 1*.

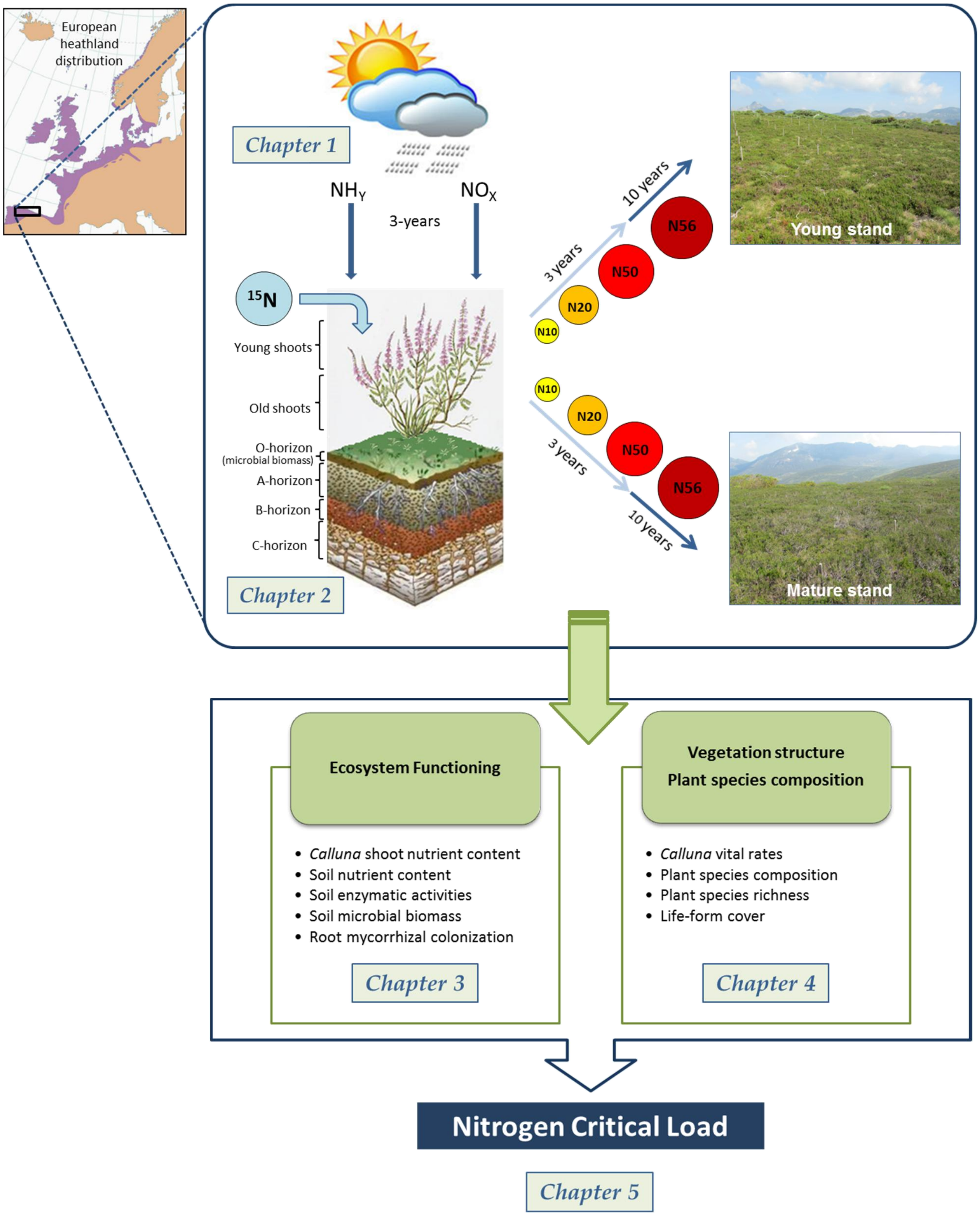
2. To quantify N sequestration and N allocation patterns among the different compartments of *Calluna vulgaris* heathlands (vegetation, soil, soil microbial biomass, and leaching losses). *Chapter 2*.

3. To evaluate the effects of increased N loads on soil chemical properties, soil enzymatic activities, soil microbial biomass nutrient content, plant mycorrhizal colonization, and plant and litter nutrient chemistry, mediated by two *Calluna vulgaris* heathland life-cycle stages (young *versus* mature stands). *Chapter 3*.

4. To analyze the effects of enhanced N loads on the vegetation structure and plant community composition in montane *Calluna vulgaris* heathlands mediated by two life cycle stages (young *versus* mature stands). *Chapter 4*.

5. To estimate the empirical critical loads for N deposition in both young and mature montane heathlands based on harmful changes in the plant community composition, vegetation structure and ecosystem functioning. *Chapter 5*.

Thesis outline



Study area

The study area is located in the Cantabrian Mountains (North-Western Spain) (Fig. 5), which are on the boundary between the Mediterranean and Atlantic/Eurosiberian biogeographical regions. Several heathland patches dominated by the dwarf-shrub *Calluna vulgaris* were selected along the southern slope of the Cantabrian Mountains (Province of León) as study sites: La Majúa, 1770 m a.s.l.; San Isidro, 1636 m a.s.l.; Riopinos I, 1653 m a.s.l.; and Riopinos II, 1567 m a.s.l. (Figs. 5 and 6). These sites have a Eurosiberian climate characterized by a dry period of less than 2 months in summer and covered by snow during late-autumn, winter, and early spring. The length of the growing season ranges from May to October. The highest rates of precipitation during the growing season occur in mid-autumn (October), whereas 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), and characterized by a sandy texture, high acidity (3.9 ± 0.14; deionized water), low fertility, and a thin humus layer (2-4 cm) (Marcos et al., 2009). This soil is covered by a shallow litter layer (0-3 cm). The plant community is dominated by *Calluna vulgaris* (L.) Hull (>75% cover), with *Erica tetralix* L. and *Vaccinium myrtillus* L. as the main accompanying species (Calvo et al., 2005).

These heathlands have been traditionally managed through grazing by transhumant flocks. Thus, they were regularly burnt in order to provide livestock pastures (Calvo et al., 2005, 2007; Morán-Ordóñez et al., 2013) (Fig. 7). These traditional management practices have contributed to maintaining heathland ecosystems with low-nutrient status (Marcos et al., 2009) by the removal of excess nutrient inputs from atmospheric depositions. However, progressive land abandonment in recent decades has determined the ageing of heathland vegetation due to the absence of management practices (Morán-Ordóñez et al., 2013), reaching the mature- and degenerate-phase of the heathland life-cycle (> 40 year-old) (Marcos et al., 2009), or even being replaced by the later successional stage of shrubland vegetation (Morán-Ordóñez et al., 2011). In recent decades, increasing atmospheric N depositions represent one of the most serious threats for these montane heathlands (García-Gómez et al., 2014).

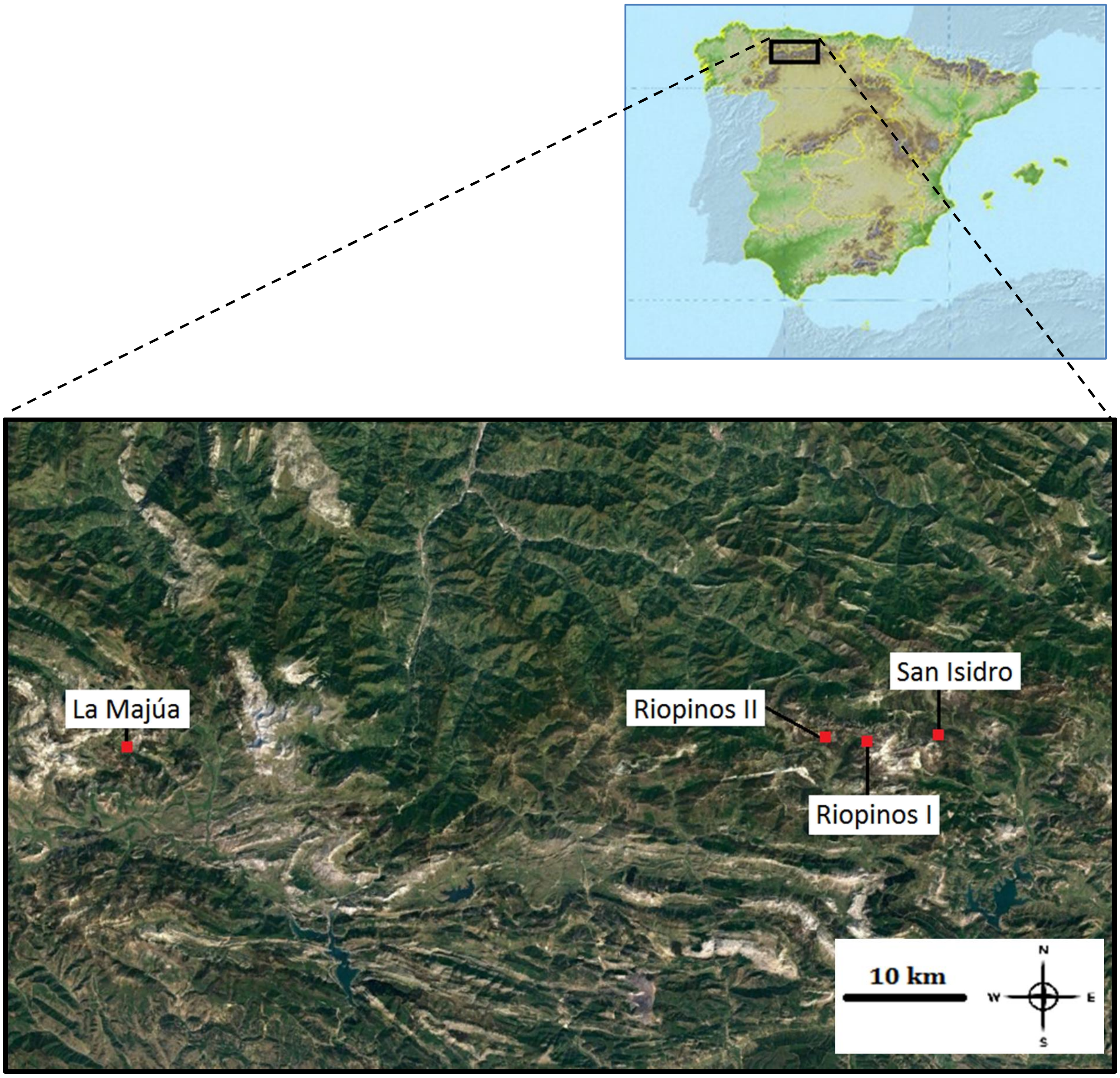


Figure 5. Location of study sites within Cantabrian Mountains range (NW Spain).

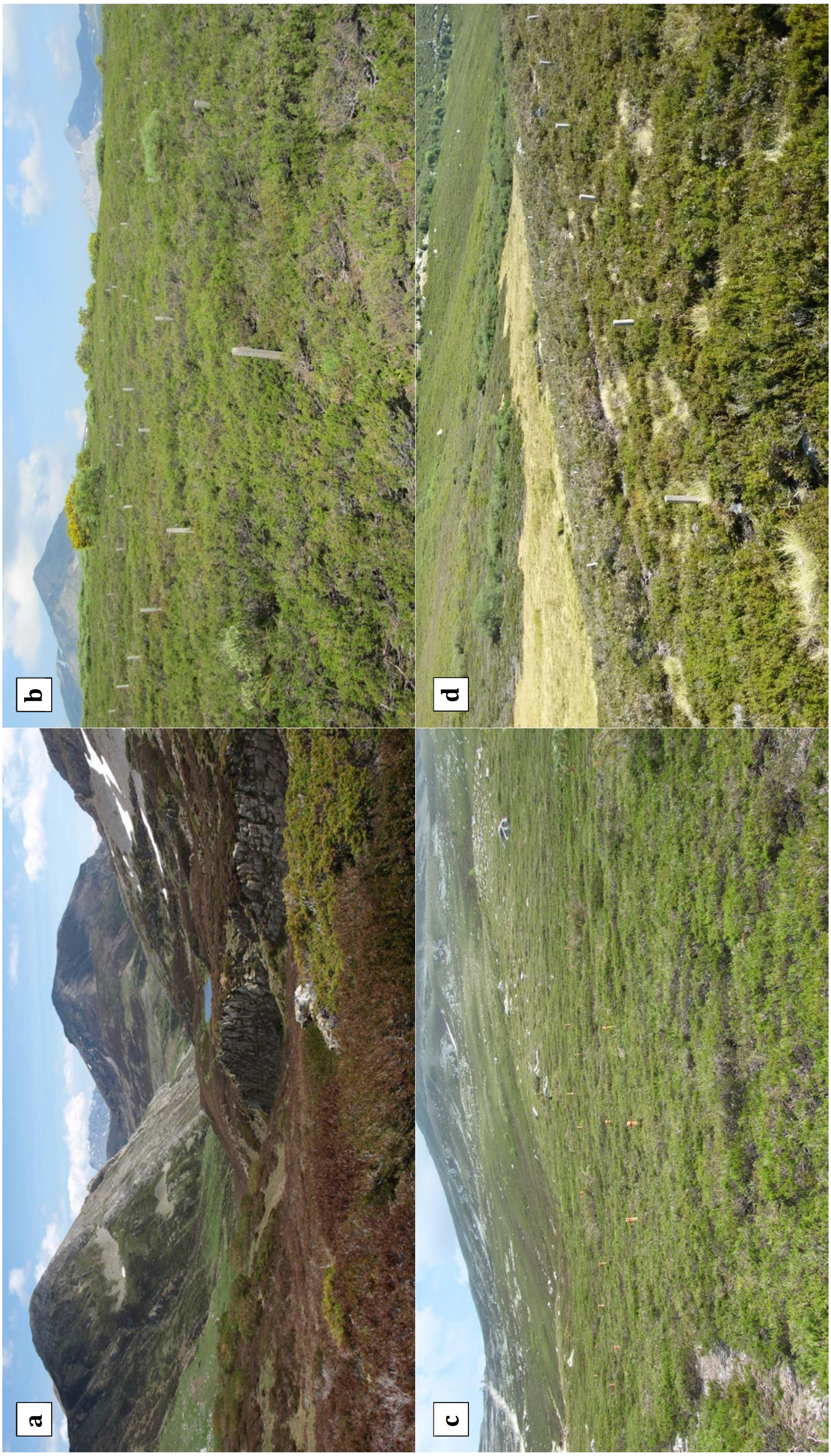


Figure 6. Heathland study sites: (a) La Majúa, (b) San Isidro, (c) Riopinos I, and (d) Riopinos II.

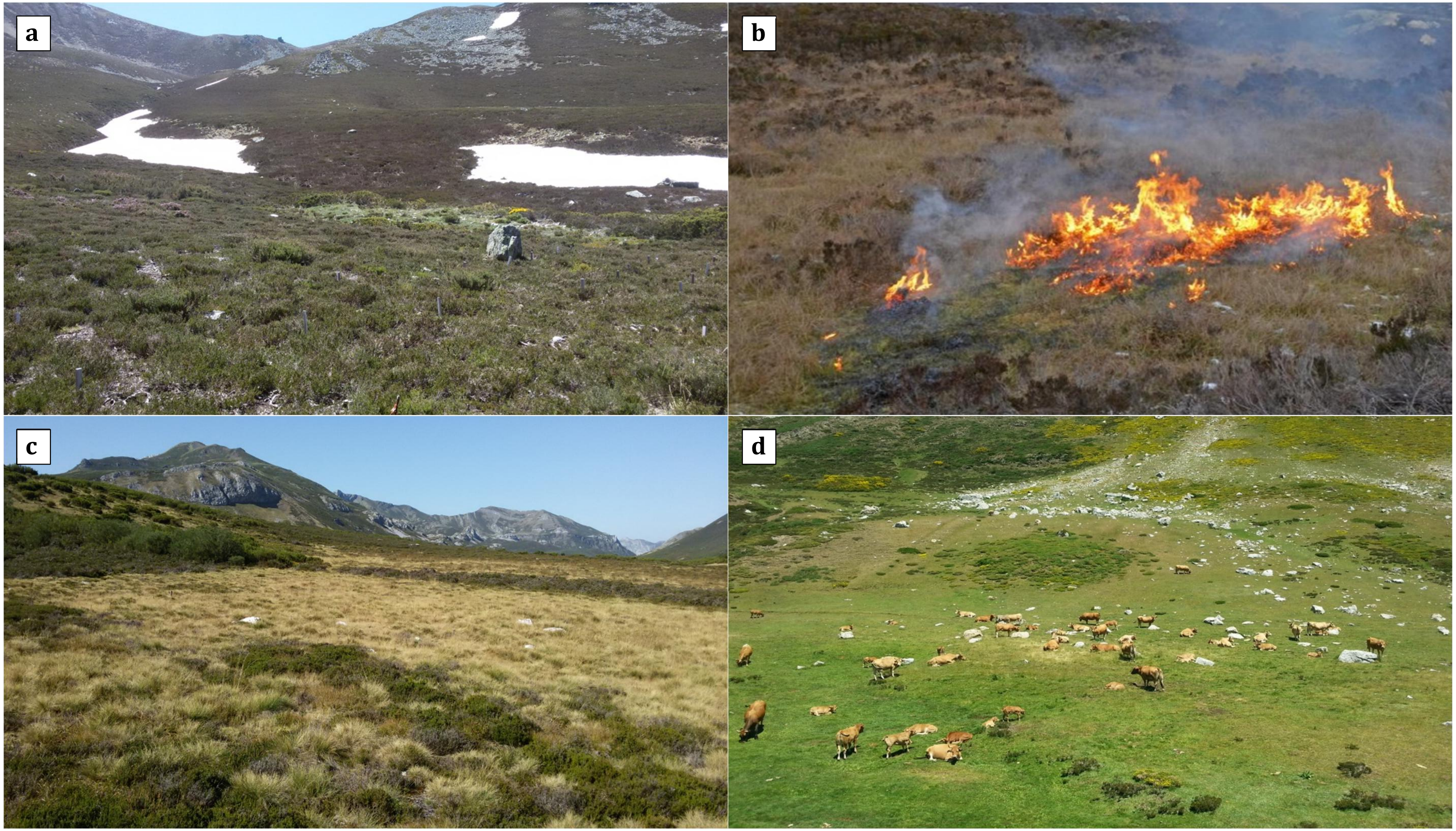


Figure 7. (a) Heathlands partially covered by snow in early June in Riopinos I; (b) traditional heathland management practice like burning; (c) 7-year-old young heathlands resulted from burning management in Riopinos II; and (d) pasture patches created by cattle livestock in La Majúa heathlands.

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CHAPTER 1

Bulk deposition of atmospheric inorganic nitrogen in mountainous heathland ecosystems in North-Western Spain [1]

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[1] The content of this chapter is based on the article:

Calvo-Fernández, J., Marcos, E., Calvo, L., 2017. Bulk deposition of atmospheric inorganic nitrogen in mountainous heathland ecosystems in North-Western Spain. Atmospheric Research 183, 237-244. http://doi.org/10.1016/j.atmosres.2016.09.006.

Abstract

Nitrogen (N) deposition has been identified as one of the main traits of terrestrial ecosystems, affecting their structure and functioning. However, few studies have been developed under natural field conditions to evaluate the amount of N deposition in low nutrient status heathland ecosystems. Therefore, a field experiment was carried out to investigate the bulk inorganic N inputs in mountainous heathlands of North-Western Spain. Two study sites (La Majúa and San Isidro) were selected on the south side of the Cantabrian Mountains, as a representative monitoring N-sensitive ecosystems. Three replicated bulk collectors and one rain gauge were installed at each study site to collect monthly bulk deposition samples over three-year period (2011-2014). Bulk inorganic N deposition was different between the study sites (2.81 kg N ha^{-1} yr^{-1} in La Majúa and 4.56 kg N ha^{-1} yr^{-1} in San Isidro), but showed the same seasonal dynamic, with higher N deposition rate in the wet period (October to April) compared to the dry period (May to September). Annual bulk NO_3^- deposition was comparable to annual bulk NH_4^+ deposition in La Majúa (1.42 *vs.* 1.39 kg N ha^{-1} yr^{-1}), and was higher in San Isidro (2.89 *vs.* 1.67 kg N ha^{-1} yr^{-1}). San Isidro displayed a characteristic bulk NH_4^+/NO_3^- deposition ratio below 1 of industrialized areas (0.58), while La Majúa displayed a bulk NH_4^+/NO_3^- deposition ratio close to 1 (0.98), distinctive of an intermediate situation between industrialized and agricultural areas. Total bulk inorganic N depositions observed in the present field study are consistent with the modelled estimation of N depositions for North-Western Spain, but only San Isidro was consistent with the estimated dominance of oxidized N over reduced N.

1.1. Introduction

Anthropogenic activities have contributed, mainly since the 1970s, to a significant increase in reactive nitrogen (Nr), including aerosols and particulate material into the atmosphere (Galloway et al., 2014), which are generated from the emissions of oxidized NO_Y (NO_X, HNO_3, N_2O_5, particulate NO_3^-, organic nitrates…) and reduced NH_X (NH_3 and particulate NH_4^+) compounds (Galloway et al., 2004). However, a different tendency compared to this global dynamic was displayed in the analysis of a shorter time period in Spain, in which decreases of 41% and 11% have been observed for NO_X and NH_3 emissions from 1990 to 2013 (MAGRAMA, 2015a). The amounts of these atmospheric emissions of NO_X, NH_3 and NO_3^- have been related to the deposition rates of NO_Y and NH_X (Aguillaume et al., 2016). Estimates of global total N depositions in 2001 were also comparable to the global total N emissions for the same year (106.3 vs. 105.1 Tg N; Vet et al., 2014). Predictions point out that global NO_Y and NH_X depositions will increase from the 1990s to 2050 (Galloway et al., 2004), although in developed areas such as Europe a decrease in N deposition of about 25% has been observed from 1990 to 2009 (Tørseth et al., 2012). In Spain, atmospheric N depositions are lower than values recorded in central Europe (Lorenz and Becher, 2012), with estimates of current rates being 12-23 kg N ha^{-1} yr^{-1} for central Europe and 3-15 kg N ha^{-1} yr^{-1} for Spain (EMEP, 2015). In addition, oxidized N deposition tends to dominate over reduced N in Spain (Fagerli et al., 2006), even after the decrease in NO_Y and NH_X depositions observed in mountainous areas of northern Spain in response to the lessening in N emissions in recent years (Izquierdo and Avila, 2012). These atmospheric N depositions depend mainly on meteorological factors such as prevailing wind direction and wind speed (Pineda Rojas and Venegas, 2010; Gómez-Carracedo et al., 2015; Javid et al., 2015), as well as the type and distribution of N emission sources (Celle-Jeanton et al., 2009; Calvo et al., 2010; Niu et al., 2014). In Spain, the sources of atmospheric NH_3 in 2012 were 92% from the volatilization of NH_3 from agricultural and farming activities (EEA, 2014), and the sources of NO_X were mainly from combustion processes of industrial activities (57%) and road traffic (34%) (EEA, 2014).

In recent years, new environmental legislation on emission control (Castellanos and Boersma, 2012; Cuevas et al., 2014) and land abandonment in mountainous systems (Morán-Ordóñez et al., 2013) have led to a reduction in N depositions in protected areas such as the Cantabrian Mountains (NW Spain). Updated N deposition data in North Western Spain, and in particular for these protected areas are necessary in order to identify whether their natural ecosystems could be receiving N deposition rates above their tolerance threshold (Bleeker et al., 2011). One of the most representative ecosystems in the mountains of North-Western Spain is *Calluna-vulgaris*-heathland, which represents a habitat of high conservation importance at European level (Habitats Directive 92/43/EEC). These *Calluna* heathlands are adapted to low-N conditions (Calvo-Fernández et al., 2015) and the knowledge of N deposition rates in these N-sensitive ecosystems could be necessary to apply appropriate management strategies (Marcos et al., 2003; Calvo et al., 2005, 2007; Boutin et al., 2015). However, only modelled N deposition data for the last few years are available for the mountainous systems of North-Western Spain, with a lack of field measured data in this area (Gómez-Carracedo et al., 2015). Besides, modelled N deposition data should be applied with caution in studies on a small regional scale and in regions with complex topography and the influence of local emissions (García-Gómez et al., 2014), since they cannot be accurate (Im et al., 2013). Boutin et al. (2015) found that mountainous areas could be more threatened by N depositions than the estimates shown by N deposition models, mainly due to the orographic scavenging effect (Cape et al., 2015).

The main aim of this article was to assess the field measured bulk inorganic N depositions in N-sensitive ecosystems of North-Western Spain in order to find seasonal dynamics of the N deposition rate. We also proposed quantifying the different inorganic N chemical forms in bulk deposition (oxidized/reduced). Finally, we proposed to compare our results about N deposition rates with the values obtained for predictive models of N deposition for our study area.

We hypothesized that the frequency and intensity of precipitation are important factors determining N deposition rates, whereby we expect to found elevated inorganic N deposition rates associated with precipitation periods (Liang et al., 2015; Zhan et al., 2015; Izquieta-Rojano et al., 2016). We also hypothesized that oxidized inorganic N deposition is higher than reduced inorganic N deposition, according to modelled N deposition data for North-Western Spain (García-Gómez et al., 2014).

1.2. Materials and methods

1.2.1. Study area and monitoring sites

The study area is located in the Cantabrian Mountains range (NW Spain). Two study sites were selected, situated 90 km apart from each other (Fig. 1.1). La Majúa is located at the top of a valley (1770 m a.s.l., 43°01’N, 6°05’W) within the Babia Biosphere Reserve and San Isidro is located in a mountain pass (1636 m a.s.l., 43°03’N, 5°21’W) at the western limit of the Picos de Europa Regional Park. Both study sites are within

Calluna-heathland areas of North-Western Spain, which represent the southern distribution range of these heathlands in Europe. The study area has a Eurosiberian climate. Mean annual temperature is 8.9 °C for La Majúa and 5.5 °C for San Isidro. Prevailing winds are from north to northeast in La Majúa and from west to west-northwest in San Isidro (Fig. 1.2). Both study sites are located near mountain passes, which connect the air masses of the northern and southern slopes of the Cantabrian Mountains range, although prevailing northern air masses in San Isidro come from the west due to the orographic conditions of this mountain pass.

1.2.2. Potential sources of N emissions in the study area

The study area is a mountainous system mainly dominated by shrub, pastureland, and forest land uses (Fig. 1.1). Scattered coal mines are located within the study area, using the extracted coal for combustion processes of a variety of industrial activities situated in lowlands. Prevailing winds in the study sites are from the highly industrialized and populated area of central-northern Asturias (North Spain), where are located several NO_Y emission sources such as coal power plants, cement factories, metal smelter plants and intense road traffic. Besides, San Isidro study site is located < 1 km from an opencast talc mine, which is an important source of NO_Y emissions due to blasting operations associated with mineral extraction processes. The study area is characterized by scarce NH_X emission sources, although La Majúa is surrounded by larger areas of pasturelands and croplands compared to San Isidro, associated with volatilization of NH_X from livestock excreta and nitrogenous fertilizers, respectively.

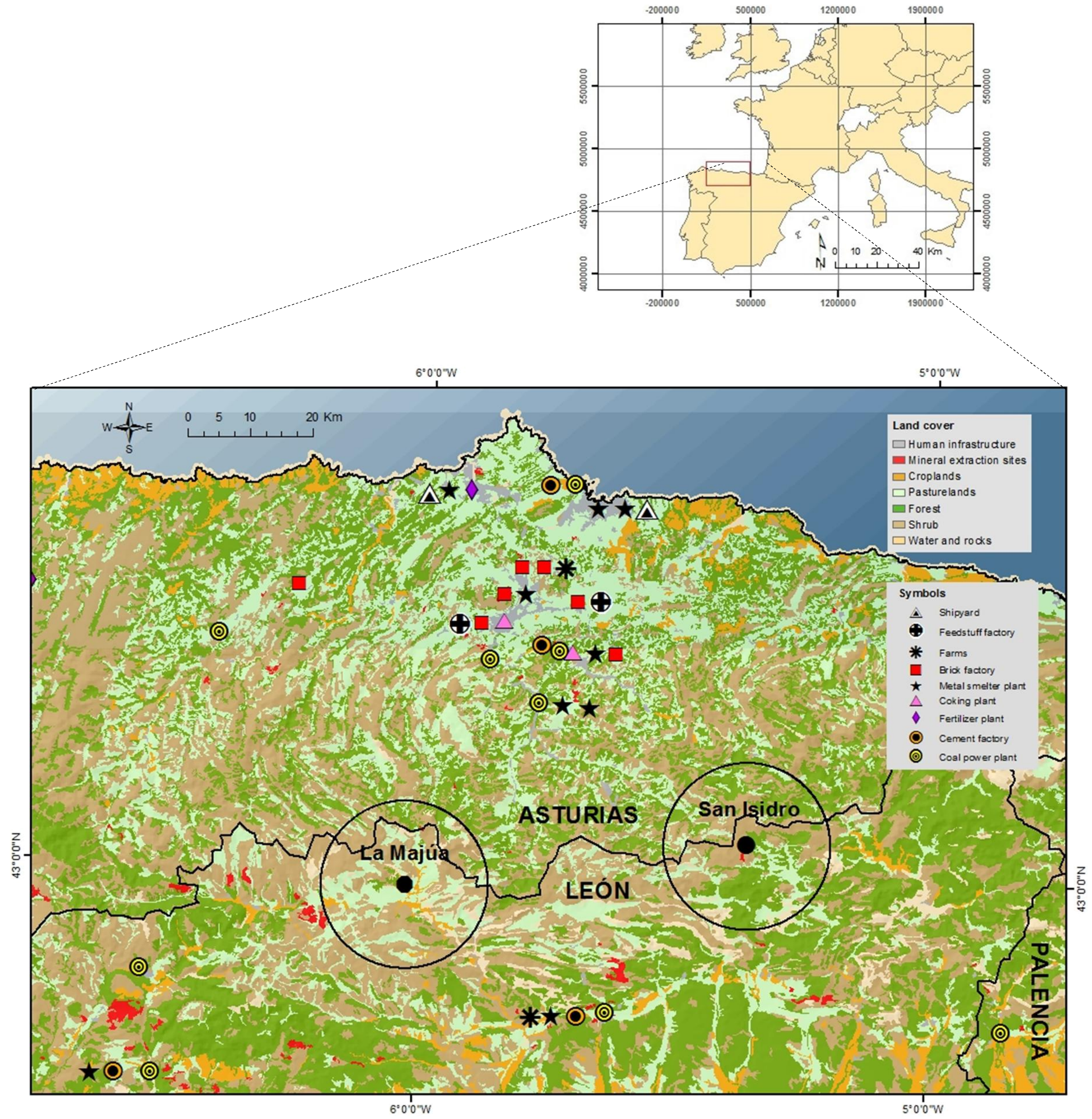

Figure 1.1. Location and potential N emission sources surrounding La Majúa and San Isidro study sites. The map represents a reclassified CORINE Land Cover 2006 in seven categories (http://centrodedescargas.cnig.es/CentroDescargas/) and the main stationary N emission sources (http://www.prtr-es.es/Informes/InventarioInstalacionesIPPC.aspx) using ArcGis v.10.3.

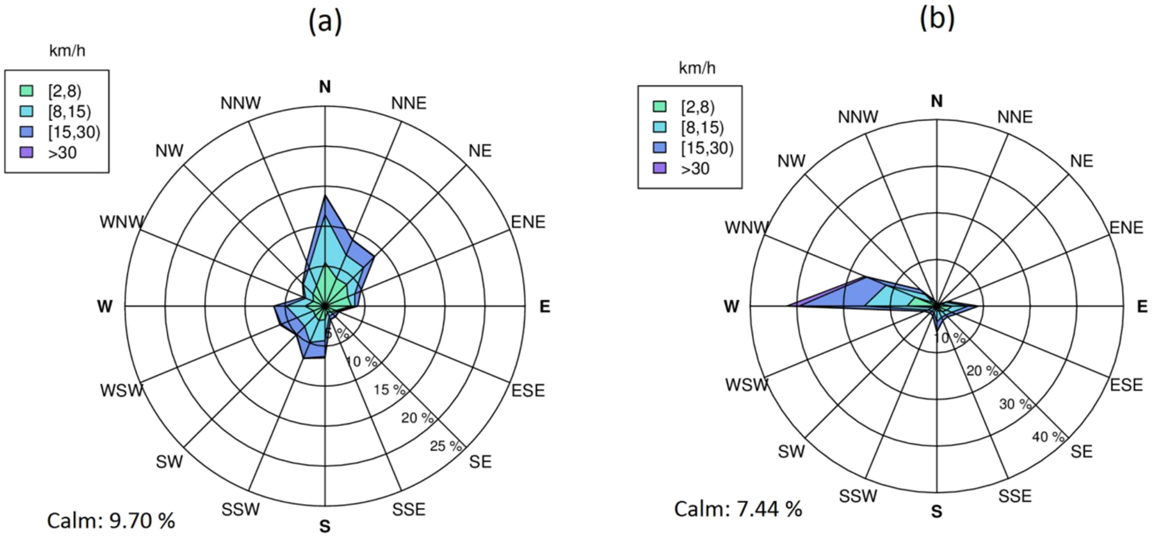

Figure 1.2. Wind roses for (a) Miñera de Luna (16 km from La Majúa) and (b) Puerto de San Isidro. They represent the wind proportion for each direction (%) and the speed range for each direction (km h^{-1}). They also show the windless period (%). Data are from 2009 to 2011 in La Majúa and from 2000 to 2011 in Puerto de San Isidro. Adapted from: http://www.atlas.itacyl.es.

1.2.3. Field sampling and chemical analysis

Three bulk collectors were installed at each study site on 1st July 2011 in open areas. We used the same type of bulk collectors used by Izquierdo and Avila (2012), which consisted of an acid-washed PVC bottle (500 ml) coupled to an acid-washed PVC funnel (12 cm diameter; 113 cm^2 horizontal interception surface). The 500 ml PVC bottle was protected from direct sunlight and biological transformations inside a PVC opaque tube. The PVC funnel was covered by a 1 mm pore size mesh to avoid contamination by insects, debris and other contaminants. Each bulk collector was supported by a metal bar placed above a grass surface at ≈ 1m height (to avoid ground contamination).

Besides, one Hellmann rain gauge (200 cm^2 collection area) was installed at each study site in order to measure the amount of precipitation.

Bulk precipitation samples were collected monthly basis in 38 sampling occasions at the end of each month from July 2011 to August 2014. After each sampling, the bulk collectors were washed and rinsed with deionized water and dried. These samples were transported in dark conditions in order to prevent any sunlight effects. Prior to analyses, the samples were filtered through a 25mm Ø cellulose acetate membrane filter (0.45 μm pore size). Ammonium concentration was analysed using the salicylate method (Reardon et al., 1966) less than 24 hours after sample collection, with a detection limit of 0.001 mg L^{-1} (Spectrophotometer UV-1700 PharmaSpec, Shimadzu, Kyoto, Japan). Nitrate concentration was determined by ion chromatography (850 Professional IC, Herisau, Switzerland) according to Tabatabai and Dick (1983) from filtered samples stored in a freezer, with a detection limit of 0.005 mg L^{-1}.

1.2.4. Calculations

Volume-weighted mean monthly and annual of NO_3^--N and NH_4^+-N concentrations in bulk precipitation (henceforth referred as NO_3^- and NH_4^+ concentrations respectively) for each study site were calculated separately with the following equation (Zhao et al., 2009):

$$C = \sum_{i=1}^{n} Ci \; x \; Li \; / \sum_{i=1}^{n} Li, \qquad (1)$$

where C refers to volume-weighted mean of NO_3^- or NH_4^+ concentration in bulk precipitation (mg N L^{-1}); Ci is the NO_3^- or NH_4^+ concentration in bulk precipitation for each individual sample (mg N L^{-1}); Li is the amount of precipitation corresponding to each sample (mm); n refers to the number of samples.

Mean monthly of NO_3^--N and NH_4^+-N deposition rates (henceforth referred as NO_3^- and NH_4^+ depositions respectively) for each study site were calculated as follows (Zhao et al., 2009):

$$D = Ci \; x \; Li \; x \; 0.01, \tag{2}$$

where D refers to mean monthly of bulk NO_3^- or NH_4^+ deposition (kg N ha^{-1} $month^{-1}$); Ci is the volume-weighted mean monthly of NO_3^- or NH_4^+ concentration in bulk precipitation (mg N L^{-1}); Li is the mean monthly of precipitation (mm).

Annual bulk NO_3 and NH_4^+ depositions (kg N ha^{-1} yr^{-1}) at each study site was also calculated as the sum of mean monthly of bulk depositions.

NH_4^+/NO_3^- ratio was also calculated from means of monthly and annual of bulk NO_3^- and NH_4^+ depositions for each study site.

1.2.5. Statistical analysis

Monthly differences in NO_3^- and NH_4^+ concentrations, bulk NO_3^- and NH_4^+ depositions, and NH_4^+/NO_3^- ratio were tested using two-way repeated measures ANOVA, with time as the repeated measure. A Pearson correlation was used to analyse the relationship among monthly precipitation with respect to NO_3^- and NH_4^+ concentrations, bulk NO_3^- and NH_4^+ depositions, and NH_4^+/NO_3^- ratio. In order to test if NH_4^+/NO_3^- ratio was different from 1, we used one sample *t*-test. All statistical analyses were performed using SPSS v.20.0 (SPSS Inc., Chicago, IL, USA).

1.3. Results

1.3.1. *NO_3^- and NH_4^+ concentrations in bulk precipitation*

Mean annual rainfall (2011-2014) was 858 mm (608 to 1059 mm) in La Majúa and 1645 mm (1608 to 1712 mm) in San Isidro, although unevenly distributed throughout the year (Fig. 1.3). We observed a high precipitation period from October to April (726 and 1379 mm for La Majúa and San Isidro respectively) and a low one from May to September (132 and 266 mm for La Majúa and San Isidro respectively).

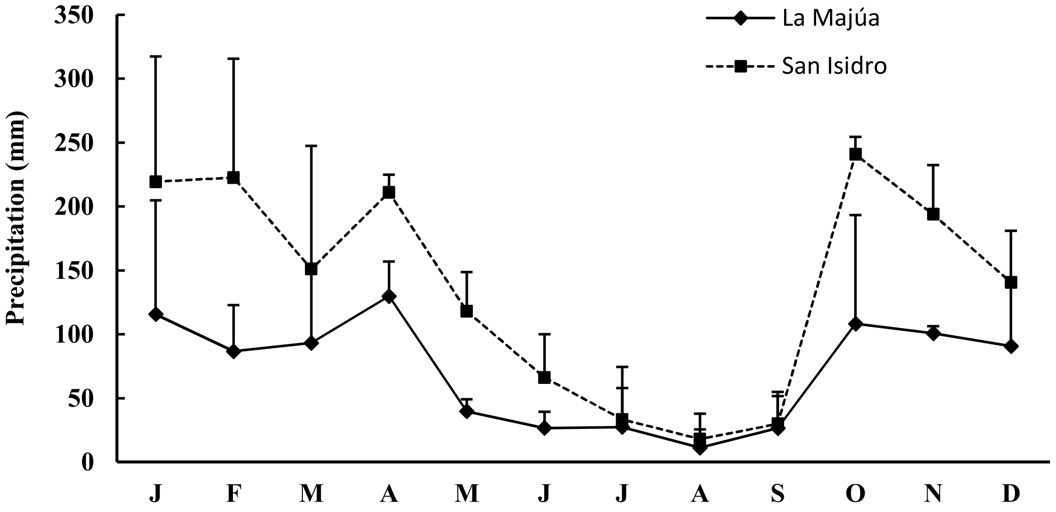

Figure 1.3. Mean monthly and standard deviation of precipitation (mm) in La Majúa and San Isidro.

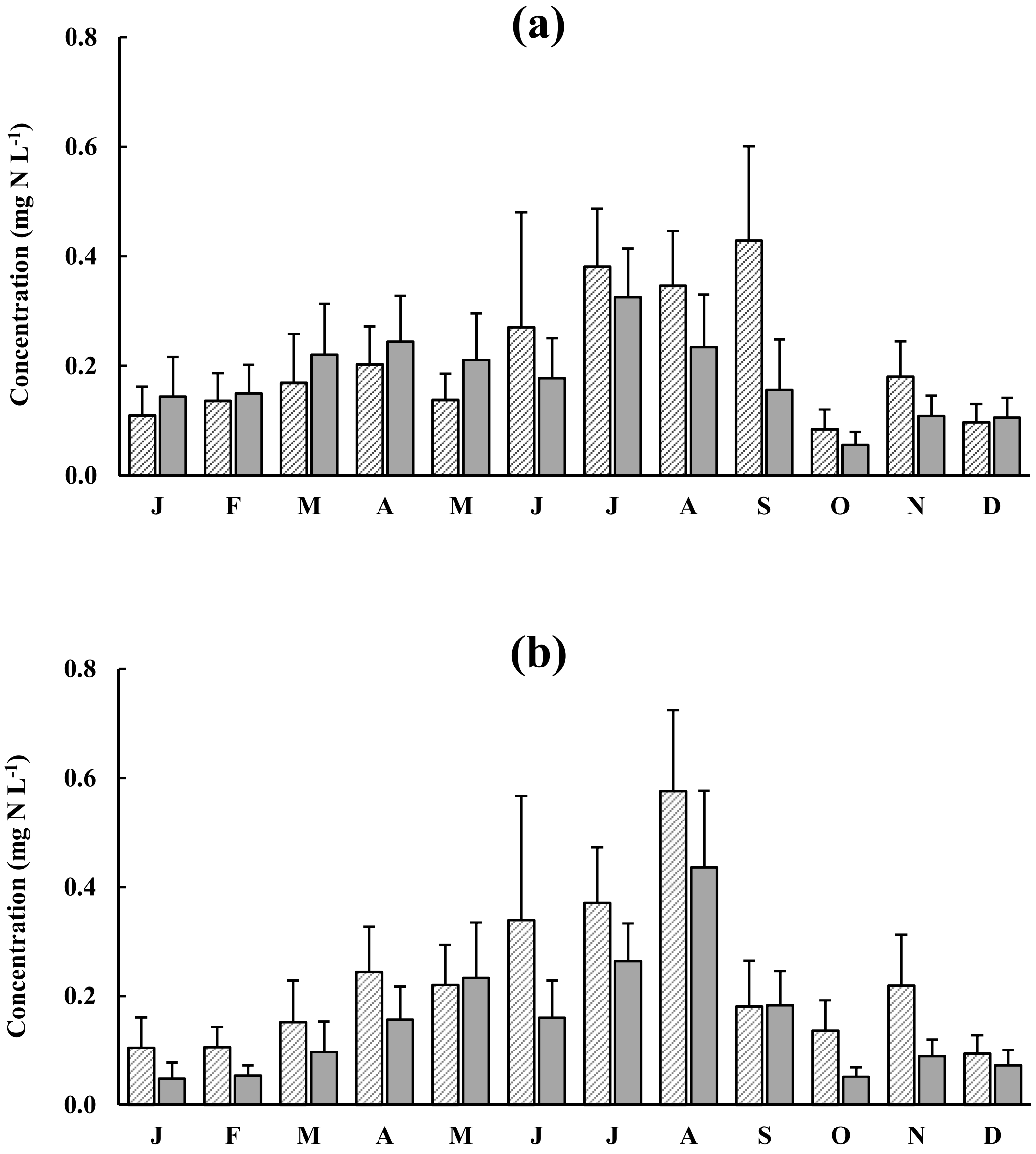

Figure 1.4. Volume-weighted mean monthly and standard deviation of NO_3^- concentrations in bulk precipitation (expressed as hatched bars; in mg N L^{-1}) and volume-weighted mean monthly and standard deviation of NH_4^+ concentrations in bulk precipitation (expressed as filled bars; in mg N L^{-1}): (a) La Majúa and (b) San Isidro.

Volume-weighted mean annual of NO_3^- concentration in bulk precipitation was higher than NH_4^+ (0.171 ± 0.039 and 0.123 ± 0.029 mg N L^{-1} respectively; $F_{(1,8)} = 42.577$; $p<0.05$); but both N compounds are significantly correlated between themselves in bulk precipitation ($r^2 = 0.79$; $p<0.01$). A seasonal dynamic with significant difference in time was observed for NO_3^- concentration in bulk precipitation ($F_{(11,44)} = 40.384$; $p<0.05$), with the highest values reached during summer months (June, July, August and September) (Fig. 1.4). NH_4^+ concentration also showed a seasonal dynamic ($F_{(11,44)} = 8.206$; $p<0.05$), with the highest values reached during spring and summer months (Fig. 1.4). It was observed that NO_3^- and NH_4^+ concentrations were negatively correlated with precipitation rates ($r^2 = -0.65$ and $r^2 = -0.59$, respectively; $p<0.01$). There was no significant difference between the study sites in the volume-weighted mean annual of NO_3^- concentration in bulk precipitation. On the contrary, the volume-weighted mean annual of NH_4^+ concentration was significantly higher ($F_{(1,4)} = 10.537$; $p<0.05$) in La Majúa (0.160 ± 0.051 mg N L^{-1}) than San Isidro (0.104 ± 0.037 mg N L^{-1}).

1.3.2. Bulk inorganic N depositions

Mean annual bulk inorganic N deposition was 2.81 kg N ha^{-1} yr^{-1} and 4.56 kg N ha^{-1} yr^{-1} in La Majúa and San Isidro, respectively. Annual bulk NO_3^- deposition was higher than NH_4^+ (1.42 *vs.* 1.39 kg N ha^{-1} yr^{-1} and 2.89 *vs.* 1.67 kg N ha^{-1} yr^{-1} for La Majúa and San Isidro, respectively); but this difference was only significant in San Isidro ($F_{(1,4)} = 37.229$; $p<0.05$). Both NO_3^- and NH_4^+ are significantly correlated between themselves in bulk deposition ($r^2 = 0.63$; $p<0.01$). Monthly differences in bulk NO_3^- ($F_{(11,44)} = 52.869$; $p<0.05$) and NH_4^+ depositions ($F_{(11,44)} = 10.273$; $p<0.05$) were found, with the highest deposition rates in April and the lowest in August or September (Fig. 1.5). Precipitation is significantly correlated with bulk NO_3^- ($r^2 = 0.56$; $p<0.01$) and NH_4^+ ($r^2 = 0.46$; $p<0.01$) depositions, displaying higher bulk NO_3^- and NH_4^+ depositions during the wet period from October to April compared to the dry period from May to September (Table 1.1). Also, there was higher bulk NO_3^- deposition in San Isidro compared to La Majúa ($F_{(1,4)}$ = 115.622; $p<0.05$), except for September (Fig. 1.5). However, no significant difference was found for bulk NH_4^+ deposition between the two study sites.

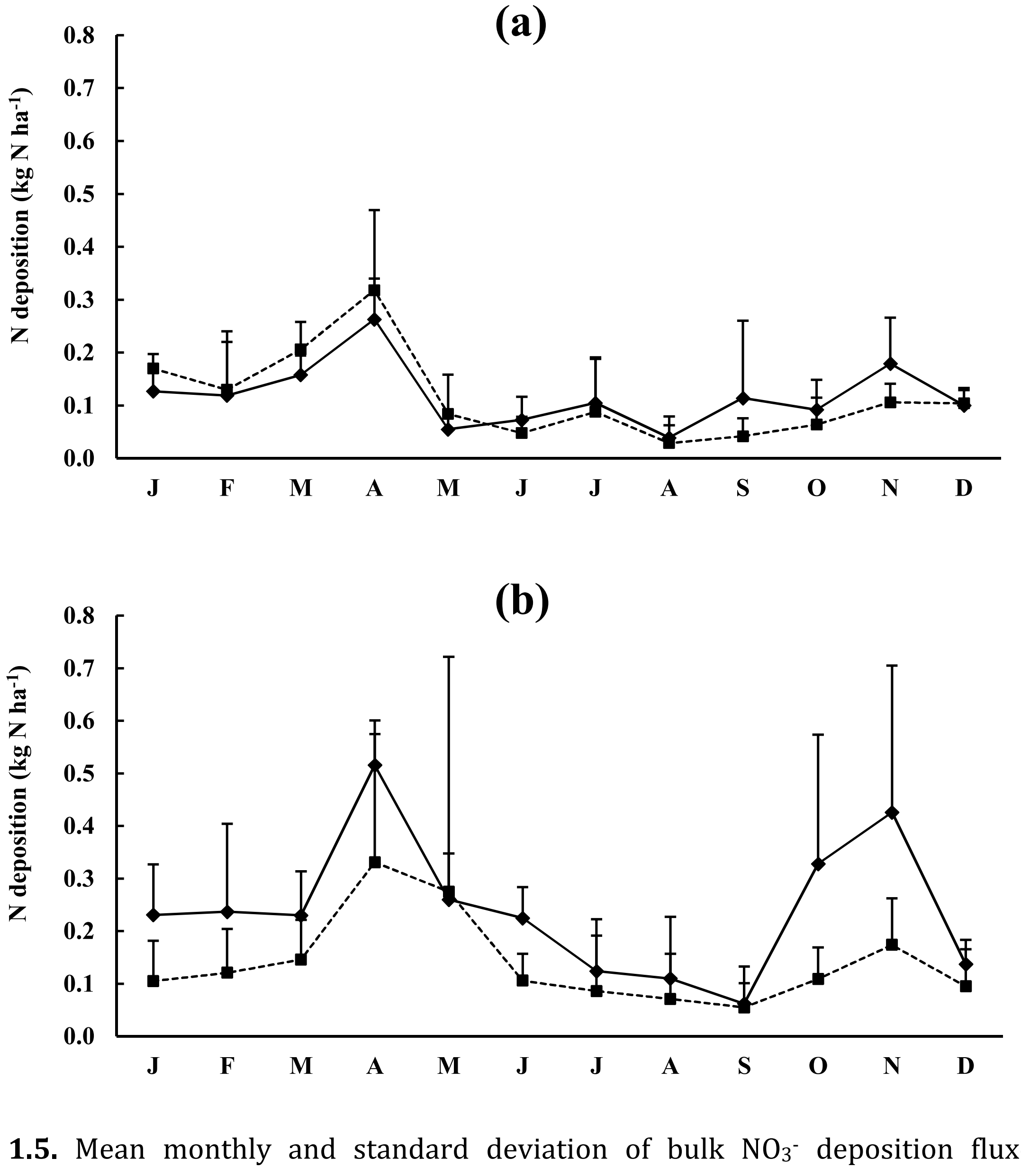

Figure 1.5. Mean monthly and standard deviation of bulk NO_3^- deposition fluxes (expressed as solid line; in kg N ha^{-1} month^{-1}) and bulk NH_4^+ deposition fluxes (expressed as broken line; in kg N ha^{-1} month^{-1}): (a) La Majúa and (b) San Isidro.

Table 1.1. Mean bulk NO_3^- and NH_4^+ deposition fluxes (kg N ha^{-1}) for the wet period (October to April) and the dry period (May to September) in La Majúa and San Isidro.

	La Majúa		San Isidro	
	NO_3^-	NH_4^+	NO_3^-	NH_4^+
Wet period	1.03	1.10	2.11	1.08
Dry period	0.39	0.29	0.78	0.59

1.3.3. NH_4^+/NO_3^- ratio in bulk deposition

Significant differences over time in the study sites were found for the NH_4^+/NO_3^- ratio in bulk deposition ($F_{(11,44)} = 2.420$; $p<0.05$). The highest NH_4^+/NO_3^- ratio was observed in May at both study sites (1.52 and 1.06 for La Majúa and San Isidro respectively), and the lowest in September in La Majúa and in October in San Isidro (Table 1.2). Annual NH_4^+/NO_3^- ratio was higher in La Majúa than San Isidro, with values of 0.98 and 0.58, respectively ($F_{(1,4)} = 15.689$; $p<0.05$). Only annual NH_4^+/NO_3^- ratio was significantly different from 1 in San Isidro ($p<0.05$).

Table 1.2. NH_4^+/NO_3^- ratio in bulk deposition for La Majúa and San Isidro. Data are means of monthly and annual NH_4^+/NO_3^- ratio with minimum-maximum values in parentheses.

	La Majúa		San Isidro	
January	1.31	(1.27-1.51)	0.45	(0.42-0.76)
February	1.10	(0.99-1.46)	0.51	(0.49-0.61)
March	1.30	(0.94-1.24)	0.64	(0.60-0.88)
April	1.21	(1.08-1.32)	0.64	(0.50-0.74)
May	1.52	(0.94-2.25)	1.06	(0.40-2.32)
June	0.66	(0.57-0.79)	0.47	(0.43-0.58)
July	0.85	(0.71-0.96)	0.71	(0.64-0.83)
August	0.68	(0.39-0.92)	0.76	(0.66-1.03)
September	0.36	(0.22-0.51)	1.01	(0.83-1.26)
October	0.66	(0.22-1.04)	0.38	(0.33-0.42)
November	0.60	(0.54-0.70)	0.41	(0.35-0.48)
December	1.08	(0.94-1.16)	0.78	(0.56-0.98)
Annual	0.98	(0.22-2.25)	0.58	(0.33-2.32)

1.4. Discussion

Large seasonal variability of NO_3^- and NH_4^+ concentrations in bulk precipitation was found in the study area, which showed the existence of an annual cycle for oxidized and reduced N concentrations in rainwater (Avila et al., 2010; Niu et al., 2014; de Souza et al., 2015). The highest NO_3^- and NH_4^+ concentrations in bulk precipitation are related to the lowest precipitation rates during summer months (Park et al., 2002; Pineda Rojas and Venegas, 2010; Izquierdo and Avila, 2012). This can be explained because the first drops of rainfall perform an intense atmospheric N scavenging, which increases the rainwater N concentration in low rainfall events (Sanz et al., 2002; Zhang et al., 2008; Al-Khashman, 2009). We observed that NO_3^- and NH_4^+ in bulk precipitation are positively well correlated, pointing out the existence of dissolved NH_4NO_3 in precipitation from the atmosphere (Bertollini *et al.,* 2016). The presence of NH_4NO_3 in precipitation is related to volatilized fertilizers which have been dissolved in rain droplets and deposited during rainfall events (Niu *et al.,* 2014).

The NO_3^- and NH_4^+ concentrations in rainwater reflect the composition of the nitrogenous gases and aerosols in the atmosphere (Celle-Jeanton et al., 2009; Calvo et al., 2010; Niu et al., 2014). For this reason, the NH_4^+/NO_3^- ratio in precipitation is a useful value to find out the prevailing sources of N depositions in a delimited area (Li et al., 2013). Zhao et al. (2009) defined a NH_4^+/NO_3^- deposition ratio <1 within industrialized regions and >1 within intensive agricultural regions. Our field study sites showed different annual NH_4^+/NO_3^- ratios in bulk deposition, with NH_4^+/NO_3^- ratio smaller than 1 in San Isidro and equal to 1 in La Majúa. This difference in NH_4^+/ NO_3^- ratio between La Majúa and San Isidro could be explained by the diversity of N sources (Li et al., 2013), since San Isidro could be more influenced by airborne NO_X from industrialized and populated areas of the northern region of Asturias (Vedrenne et al., 2015). Besides, larger NH_4^+ fertilized pastureland and cropland areas surrounding La Majúa compared to San Isidro could have explained the same contribution of NH_Y as NO_X to total N deposition observed in La Majúa. Thus, only San Isidro was in agreement with the estimates of oxidized N dominance over reduced N obtained by N deposition models for North-Western Spain (García-Gómez et al., 2014). The inaccurate of N deposition models in La Majúa could be due to the great influence of local emissions on N deposition in those areas with complex topography (Im et al., 2013). However, land abandonment dynamic in mountainous areas of North-Western Spain observed during the last 50 years could contribute to a reduction in NH_3 emissions associated with traditional management such as livestock grazing and heath burning (Morán-Ordóñez et al., 2013), and therefore, a greater dominance of oxidized N deposition according to estimates of N deposition models.

Bulk inorganic N deposition is conditioned by several factors, such as volume of precipitation and seasonal influence of emission sources (Cape et al., 2015; Liang et al., 2015; Zhan et al., 2015; Izquieta-Rojano et al., 2016), as well as the chemical and physical N removal processes from the atmosphere (de Souza et al., 2015). Bulk NO_3^- deposition in our field study was consistent with the modelled estimation for North-Western Spain (1.5-2.0 kg N ha^{-1} yr^{-1}; Vet et al., 2014). We observed higher bulk NO_3^- deposition in the rainiest months compared to the driest summer months, pointing out that N deposition is strongly influenced by precipitation amount (Liang et al., 2015). It could be also explained because NO_2 emissions to the atmosphere in North-Western Spain are higher during winter season due to fossil fuel consumption in building heating systems (Cuevas et al., 2014; Gómez-Carracedo et al., 2015). Bulk NH_4^+ deposition in our field study was within the range of the modelled estimation for North-Western Spain (1.0-2.0 kg ha^{-1} yr^{-1}; Vet et al., 2014). The highest bulk NH_4^+ deposition in our study was obtained in April, reflecting the emissions of volatilized NH_3 from nitrogenous fertilizers spread over croplands in late-winter and spring (Asman et al., 1998; Rodà et al., 2002; Zhan et al., 2015). We also observed high bulk NH_4^+ deposition at the beginning of the wet period in October-November (as well as bulk NO_3^- deposition), reflecting a precipitation scavenging of nitrogenous gases and particles accumulated in the atmosphere during the dry period (Al-Khashman, 2009), and displaying that NO_3^- and NH_4^+ depositions are highly correlated between themselves (van den Berg et al., 2016). Total annual bulk inorganic N deposition observed in the study sites was lower compared to other mountainous areas of Spain as Pyrenees (5.42 to 10.07 kg N ha^{-1} yr^{-1}; Rodà et al., 2002; Izquierdo and Avila, 2012), which could be supported by the prevalence of unpolluted air masses from the Atlantic Ocean crossing the North-West Spain (Santos et al., 2011). Despite low rates of N deposition observed in our field study, N deposition models point out that heathlands of North-Western Spain could be receiving an exceedance above 2.5 kg N ha^{-1} yr^{-1} (García-Gómez et al., 2014) over their empirical N critical load of 10 kg N ha^{-1} yr^{-1} (Bobbink et al., 2010).

The chemical form of N inputs is an important regulating factor of plant nutrient assimilation processes in a wide variety of ecosystems (Pornon et al., 2007; Stevens et al., 2011; Harmens et al., 2014; Izquieta-Rojano et al., 2016). It has been demonstrated that NH_4^+ is more likely to be toxic for plant root assimilation than NO_3^- (Sheppard et al., 2014), hence lower NH_4^+ input respect to NO_3^- input particularly observed in San Isidro could contribute to the lessening of harmful effects on heathland vegetation. Besides,

NH_4^+ deposition has a greater impact on vegetation composition than NO_3^- deposition (van den Berg et al., 2016), since NH_4^+ deposition can lead soil acidification by the release of H^+ ions (Stevens et al., 2011); being the main pathway of biodiversity loss in ecosystems adapted to N-poor conditions (Boutin et al., 2015). This could have a great impact in mountainous heathlands of North-Western Spain, recognized as a biodiversity hotspot (Morán-Ordóñez et al., 2011). Respect to the seasonal dynamic in bulk precipitation, the high NO_3^- and NH_4^+ concentrations found during summer could affect the nutrient balance of N-poor ecosystems such as mountainous heathlands (Stevens et al., 2011; Calvo-Fernández et al., 2015), since this period is coincident with the growing season, and therefore, with the highest rates of N uptake by heathland vegetation. Previous studies have demonstrated that an increase in N depositions in heathland ecosystems could involve higher shoot N content, inducing heather defoliation by leaf beetles, such as *Lochmaea suturalis* (Cuesta et al., 2008; Torres, 2010), as well as a rise in soil nutrient content (Marcos et al., 2015).

1.5. Conclusions

Field measured bulk inorganic N deposition in North-Western Spain was evaluated in this study. Annual bulk deposition was 2.81 kg N ha^{-1} yr^{-1} and 4.56 kg N ha^{-1} yr^{-1} for La Majúa and San Isidro study sites, respectively. A seasonal dynamic was found for bulk NO_3^- and NH_4^+ depositions in the study area. This seasonal dynamic was dependent on the precipitation rate, with the highest monthly bulk NO_3^- and NH_4^+ depositions observed in the rainiest months (October to April). It was found that annual bulk NO_3^- deposition was higher than annual bulk NH_4^+ deposition in San Isidro, consistent with the observed NH_4^+/NO_3^- deposition ratio <1 distinctive of industrialized areas. However, La Majúa displayed comparable annual bulk NO_3^- and NH_4^+ deposition, distinctive of an intermediate situation between industrialized and agricultural areas. Despite lower rates of N deposition obtained in the present study regarding empirical N critical load for dry heathlands, studies about the effect of current N deposition in the structure and functioning of N-sensitive heathlands would be needed.

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CHAPTER 2

Allocation patterns of airborne nitrogen in mountainous heathlands

- A ^{15}N tracer study in the Cantabrian Mountains (NW Spain) [1]

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[1] The content of this chapter is based on the article:

Calvo-Fernández, J., Marcos, E., Calvo, L., Härdtle, W., 2015. Allocation patterns of airborne nitrogen in mountainous heathlands - A ^{15}N tracer study in the Cantabrian Mountains (NW Spain). Ecological Engineering 84, 128-135. http://doi.org/10.1016/j.ecoleng.2015.07.027.

Abstract

The fate of atmospheric N depositions in heathlands dominated by *Calluna vulgaris* (L.) Hull in the Cantabrian Mountains (NW Spain) was analysed 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.

2.1. 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; Galloway et al., 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} yr^{-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} yr^{-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} yr^{-1} (García-Gomez 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; Arino 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 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 (Aber et al., 1998).

2.2. Materials and methods

2.2.1. Study site

The study site is located on the León (south) side of the Cantabrian Mountains 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 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 *Calluna 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%.

2.2.2. Study design

Two plots of 3m x 7m were randomly selected at each study site in homogenous stands of *Calluna* in July 2011 (i.e. four replicates in total). Two 1m x 2m 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 1m to avoid contamination of the control subplot. To calculate N losses by leaching, other four 3m x 5m 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 disk (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 12V gel battery, protected inside a sealed box. Plots and lysimeters were fenced in to prevent damage by grazing animals.

2.2.3. ^{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.

2.2.4. 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 2.1) 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 analyse the soil microbial biomass 5cm x 5cm 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.

2.2.5. 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.

2.2.6. 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 hours, 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 δ^{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 δ^{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. δ^{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 δ^{15}N-NO_3^- in leachate samples (Sigman et al., 1997) and δ^{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 analysed 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 $\delta^{15}N$.

2.2.7. 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 hours. 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 $N\text{-}NO_3^-$ and $N\text{-}NH_4^+$ in the labelled lysimeters.

2.2.8. 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:

$$\delta^{15}N\ (‰) = [(Rsample\ /\ Rstandard) -1] \times 10^3, (1)$$

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). $\delta^{15}N\text{-}NO_3^-$ and $\delta^{15}N\text{-}NH_4^+$ 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 $\delta^{15}N\text{-}NO_3^-$ and $\delta^{15}N\text{-}NH_4^+$ 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:

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

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}), $\text{at.\%}^{15}N_{pool}$ is the $\text{at.\%}^{15}N$ in the N pool of labelled subplots or in leachate losses from labelled lysimeters, $\text{at.\%}^{15}N_{ref}$ is the $\text{at.\%}^{15}N$ in the N pool of control subplots or in leachate from control lysimeters, and $\text{at.\%}^{15}N_{tracer}$ is the $\text{at.\%}^{15}N$ of the added ^{15}N tracer.

2.2.9. 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).

2.3. Results

2.3.1. ^{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 2.1). ^{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 2.1). ^{15}N natural abundance of the soil microbial biomass varied between -3.81‰ and 10.85‰, with no clear temporal pattern (Table 2.1).

Table 2.1. ^{15}N natural abundances of the *Calluna* biomass, soil and soil microbial biomass in the control subplots. Data are means of $\delta^{15}N$ (‰) with (SE). 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).

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

2.3.2. ^{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. 2.1). *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. 2.1). 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 peak in week 70.

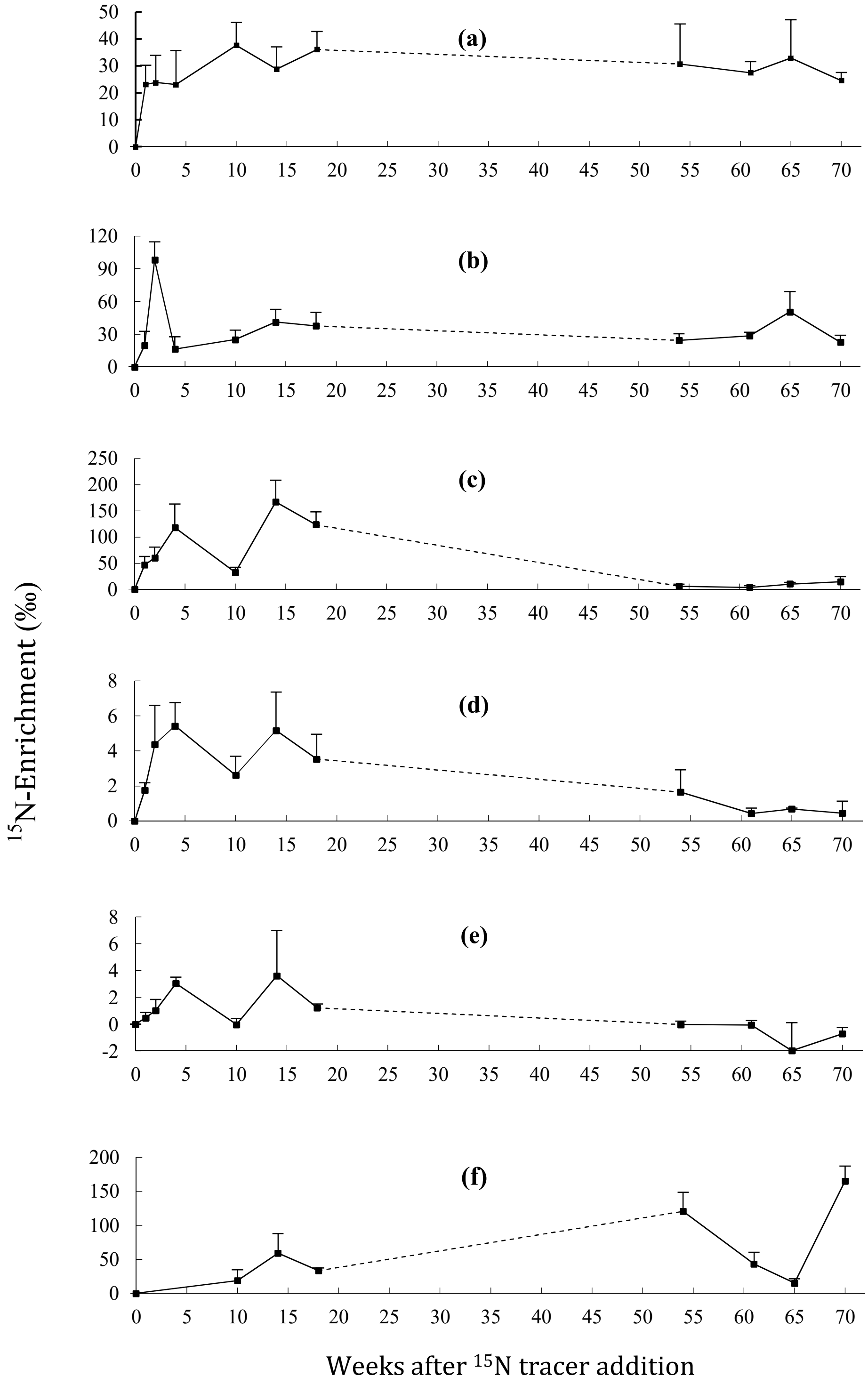

Figure 2.1. ^{15}N enrichment (‰) of the heath compartments analyzed for 10 sampling occasions (7 for microbial biomass) following ^{15}N tracer addition (weeks after ^{15}N tracer addition). (a) new shoots; (b) old shoots; (c) O-horizon; (d) A-horizon; (e) B-horizon; (f) soil microbial biomass. Broken lines between weeks 18 to 54 represent the absence of sampling in this period.

2.3.3. ^{15}N tracer recovery

Table 2.2 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.

Table 2.2. ^{15}N tracer recovery of the heath compartments analysed for two late growing season dates (November 2011 and November 2012).

		November 2011				November 2012			
Compartment		^{15}Nrec (mg N m^{-2})		% ^{15}Nrec		^{15}Nrec (mg N m^{-2})		% ^{15}Nrec	
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}Nrec) and as a percentage of total ^{15}N tracer masses (%^{15}Nrec). 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 ($p<0.05$).

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 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 2.3). ^{15}N tracer recovery in leachate was very low in both years, with higher values for $^{15}N\text{-}NO_3^-$ as compared to $^{15}N\text{-}NH_4^+$.

Table 2.3. ^{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}Nrec	g N m^{-2}	%^{15}Nrec	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.

2.3.4. *^{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. 2.2). Thus, ^{15}N losses via leaching were much lower as compared to the amount of ^{15}N tracer retained by *Calluna* shoots, soil horizons or soil microbial biomass (Table 2.2). ^{15}N losses by leaching were detectable in the fourth week after tracer addition (Fig. 2.2). No leaching losses appeared in weeks 14, 54, 61 and 65 (Fig. 2.2), which coincided with dry periods in summer (Table 2.3). We found a clear relationship between the amount of N leached and ^{15}N recovery in the leachate (Fig. 2.2). ^{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 2.3).

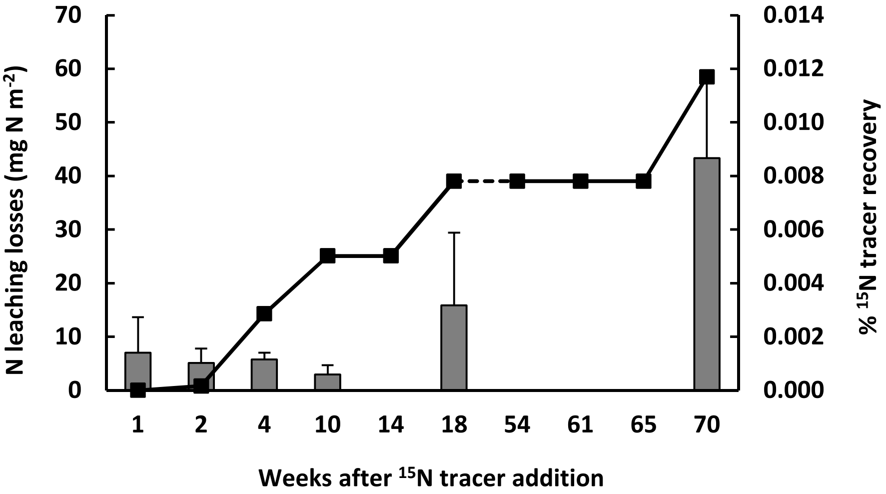

Figure 2.2. ^{15}N leaching losses expressed as a cumulative curve of ^{15}N recovery (%) compared to N leaching losses (expressed as filled bars; in mg N m^{-2}). Broken line between weeks 18 to 54 represents the absence of sampling in this period. N leaching losses and ^{15}N tracer recovery in the first two weeks are only NO_3^- data.

2.4. Discussion

2.4.1. ^{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 *Calluna 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).

2.4.2. ^{15}N leaching losses

Low N losses through leaching confirmed our hypothesis that the heathlands analysed 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).

2.4.3. 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 analysed 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.

2.5. 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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CHAPTER 3

Time- and age-related effects of experimentally simulated nitrogen deposition on the functioning of montane heathland ecosystems [1]

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[1] The content of this chapter is based on the article:

Calvo-Fernández, J., Taboada, A., Fichtner, A., Härdtle, W., Calvo, L., Marcos, E., 2017. Time- and age-related effects of experimentally simulated nitrogen deposition on the functioning of montane heathland ecosystems. Submitted to Science of the Total Environment, under second revision.

Abstract

Ecosystems adapted to low nitrogen (N) conditions such as *Calluna*-heathlands are sensitive to enhanced atmospheric N deposition that affects many aspects of ecosystem functioning. We investigated the effects of five levels of experimentally-simulated N deposition rates (i.e., N fertilization treatments: 0, 10, 20 and 50 kg N ha^{-1} yr^{-1} for 3 years, and 56 kg N ha^{-1} yr^{-1} for 10 years) on: plant, litter, microbial biomass and soil nutrient contents, soil extracellular enzyme activities, and plant root mycorrhizal colonization. The study was conducted in marginal montane *Calluna*-heathlands at different developmental stages resulting from management (young/building-phase and mature-phase). Our findings revealed that many soil properties did not show significant response to the experimental addition of N, including: total N, organic carbon (C), C:N ratio, extractable N-NO_3^-, available phosphorus (P), urease and β-glucosidase enzyme activities, and microbial biomass C and N. There was a considerable positive impact of chronic (10-year) high-N load on soil extractable N-NH_4^+, acid phosphatase enzyme activity, *Calluna* root mycorrhizal colonization, *Calluna* shoot N and P contents, and litter N content and N:P ratio. The age of heathland vegetation influenced the effects of N addition on mycorrhizal colonization, resulting in higher colonized roots in young heathlands at the control, low and medium N-input rates; and in mature ones at the high and chronically high N rates. Also, young heathlands exhibited greater soil extractable N-NO_3^-, available P, microbial biomass N, *Calluna* shoot N and P contents, and litter N content, compared to mature ones. Our results highlighted that accounting for the N-input load and duration, as well as the developmental stage of the vegetation, when assessing the effects of N fertilization is crucial to preserve the functioning of heathland ecosystems, particularly at their southern distribution limit.

3.1. Introduction

The increase in human-induced atmospheric reactive nitrogen (N) in the last century has resulted in a dramatically increase in N deposition rates (Calvo-Fernández et al., 2017), which are expected to rise in future decades (Galloway et al., 2004). Airborne N load has been identified as one of the most important drivers of biodiversity loss at a global scale (Sala et al., 2000), which in turn is expected to have negative consequences for multiple ecosystem functions. There exists compelling evidence of N-driven damage to ecosystems even at low deposition rates (Phoenix et al., 2012; Bähring et al., 2017). Moreover, chronic N load has severe impacts on many ecosystem functions when the critical N threshold is exceeded (Gao et al., 2014). These harmful effects are caused by ecosystem eutrophication and soil acidification processes (Bobbink et al., 2010; Stevens et al., 2011; Zhu et al., 2015), altering the biogeochemical cycles of N, carbon (C) and phosphorus (P) (Erisman et al., 2011).

Ecosystems adapted to low levels of nutrient availability, such as heathlands dominated by the dwarf shrub *Calluna vulgaris* (L.) Hull (henceforth referred to as *Calluna*), are particularly sensitive to airborne N deposition (Cuesta et al., 2008; Jones and Power, 2012; Southon et al., 2012; Fagúndez, 2013; Meyer-Grünefeldt et al., 2016). Both field-scale surveys and N-manipulation experiments testing the effects of a variety of N-load rates over different temporal scales have evidenced substantial N-driven changes in the composition, diversity and functioning of nutrient-poor *Calluna*-heathlands (e.g., Calvo et al., 2005, 2007; Power et al., 2006; Friedrich et al., 2011; Southon et al., 2013), threatening their persistence across Europe (Fagúndez, 2013). Moreover, several studies have evaluated the cumulative effects of N in heathland ecosystems (Johnson et al., 1998; Phoenix et al., 2012; Southon et al., 2012; among others), since chronic N load is expected to aggravate the impact of N even at low input rates (Power et al., 2006; Phoenix et al., 2012).

Increased N inputs alter a multitude of heathland characteristics such as soil and litter properties (e.g., nutrient availability, enzyme activities or microbial biomass) or plant traits [e.g., growth, flowering, tissue and litter chemistry or plant susceptibility to biotic (e.g., pathogens and pests) and abiotic (e.g., frost or drought) stressors] (Marcos et al., 2003; Jones and Power, 2012; Southon et al., 2013; Meyer-Grünefeldt et al., 2016; Taboada et al., 2016; Bähring et al., 2017). Elevated N inputs stimulate N mineralization rates (Phoenix et al., 2012), resulting in increased soil extractable N-NH_4^+ and N-NO_3^- (Southon et al., 2013; Boot et al., 2016; Song et al., 2017). This enhanced soil N availability may cause either an increase (Haugwitz et al., 2011; Du et al., 2014) or decrease (Ajwa et al., 1999; Boot et al., 2016) in the nutrient contents of the soil microbial biomass, altering the cycles of soil C and N (Ramírez et al., 2012; Contosta et al., 2015; Zhu et al., 2015), and the ericoid mycorrhizal (ERM) fungal community associated with *Calluna* (Caporn et al., 1995). Since soil microorganisms are considered the primary sources of soil enzymes, and these are involved in nutrient metabolism and decomposition processes (Ramírez et al., 2012; Sinsabaugh and Follstad, 2012; Zhu et al., 2015; Fatemi et al., 2016; Song et al., 2017), an increase in N inputs is expected to alter soil enzymatic activities such as acid phosphatase (P cycle), urease (N cycle) and β-glucosidase (C cycle) (Ajwa et al., 1999; Ochoa-Hueso et al., 2011, 2014; Jian et al., 2016). These variations very likely affect the storage, turnover and uptake of soil nutrients (Jones and Power, 2012; Cenini et al., 2016). As a result, excess N accumulation in heathland ecosystems promotes enhanced rates of nutrient uptake by *Calluna* plants and subsequent increases in foliar tissue N and P contents (Pilkington et al., 2005b;

Calvo et al., 2007; Rowe et al., 2008; Jones and Power, 2012; von Oheimb et al., 2010), as well as increases in litter N and P contents (Pilkington et al., 2005b).

Age-related differences in *Calluna* nutrient uptake and growth rate are expected to influence the impacts of N deposition on heathlands (Jones and Power, 2015; Meyer-Grünefeldt et al., 2015), but till now only a limited number of studies have assessed these effects (i.e., Britton et al., 2008; Jones and Power, 2015). European heathlands have traditionally been managed to create pastures for breeding livestock and their nutrient poor status has been preserved through practices as mowing, sod cutting and prescribed burning (Härdtle et al., 2006, 2009; Fagúndez, 2013; Jones et al., 2017), resulting in the periodic rejuvenation of heathland vegetation (Gimingham, 1972; Henning et al., 2017). In recent decades, however, land use abandonment has led to heathland management cessation and to *Calluna* plants reaching the mature or degenerate phase of development (*sensu* Gimingham, 1972; Calvo et al., 2007; Henning et al., 2017). As time progresses since the last management (e.g., prescribed burning, mowing, sod-cutting, and grazing), ageing heathland ecosystems accumulate N in soils and in the vegetation biomass (Härdtle et al., 2009; Jones and Power, 2015). Therefore, specific measures to compensate for atmospheric N deposition are required to remove the excess of N stored in the ecosystem, and thus to keep a low-N status (Calvo et al., 2005; Härdtle et al., 2006, 2009; Marcos et al., 2009; Jones et al., 2017).

In contrast to north-western (e.g., Phoenix et al., 2012; Southon et al., 2012) and central European (e.g., de Vries et al., 2009; Friedrich et al., 2011; Bähring et al., 2017) *Calluna*-heathlands, to date, only one study has been developed on the time-scale and age-related effects of enhanced N deposition in montane *Calluna*-heathlands located at the southern-most limit of their distribution range (Cantabrian Mountains, NW Spain) (i.e., plant-herbivore-predator relationships: Taboada et al., 2016). This is despite these marginal southern *Calluna*-heathlands having been found to respond differently to global change drivers (such as N deposition) as compared to central European ones (Meyer-Grünefeldt et al., 2016). In this study, we evaluated the effects of different levels of experimentally simulated N deposition on the functioning of marginal montane *Calluna*-heathlands, mediated by the age of heathland vegetation resulting from management activities (prescribed burning), with particular attention being paid to the cumulative impact of N load throughout time. Specifically, we assessed the effects of five levels of N fertilization rates (0, 10, 20 and 50 kg N ha^{-1} yr^{-1} for 3 years, and 56 kg N ha^{-1} yr^{-1} for 10 years) on: (1) soil chemical properties, (2) soil extracellular enzymatic activities, (3) soil microbial biomass C and N, (4) plant mycorrhizal colonization, (5) plant nutrient uptake, and (6) litter chemistry. To our knowledge, this is the first study that evaluates the overall impact of cumulative N loading on plant-soil-microbial-enzyme relationships in both young and mature developmental stages of European heathlands.

We hypothesize that an increase in N load will result in: (1) a subsequent increase in plant-litter-soil N and P contents due to higher nutrient accumulation and immobilization, as well as an increase in plant and litter N:P ratios (Britton et al., 2008; von Oheimb et al., 2010; Southon et al., 2013); (2) faster rates of extracellular enzymatic activities to supply higher plant and microbial nutrient demands (Ochoa-Hueso et al., 2011, 2014); (3) a rise in soil microbial biomass C and N contents (Power et al., 2006; Haugwitz et al., 2011), and (4) variations in the extent of root mycorrhizal colonization related to plant nutrient demands (Caporn et al., 1995; Rowe et al., 2008). We also hypothesize that chronic (10-year) N inputs will have a greater impact compared to short-term (3-year) N load (Phoenix et al., 2012). Furthermore, we expect that higher plant and soil microbial biomass in mature heathlands relative to young ones will have comparatively greater nutrient demands resulting from N fertilization (due to nutrient stoichiometry) (Wendling et al., 2016), which, in turn, should be mirrored by lower soil nutrient contents (Ajwa et al., 1999).

3.2. Material and methods

3.2.1. Study area

The study area is located on the southern slope of the Cantabrian Mountains range (NW Spain). We selected three representative and homogeneous *Calluna*-heathland sites situated at least 3 km apart: Riopinos I (1660 m a.s.l., 43°02'N, 5°24'W, 24 ha) is a discontinuous northern-exposed heathland on a steep slope; Riopinos II (1560 m a.s.l., 43°02'N, 5°26'W, 18 ha) is a wind-exposed heathland in a north-facing area with a low slope; San Isidro (1620 m a.s.l., 43°03'N, 5°21'W, 35 ha) is a flat and continuous heathland facing north and exposed to winds. The climate is Eurosiberian with a mean annual temperature of 5.5 °C. Mean annual precipitation is 1645 mm, unevenly distributed throughout the year, with a brief drought period during the summer months (Calvo-Fernández et al., 2017). Precipitation occurs mainly in the form of snow in late-autumn, winter and early-spring, with a snow melt period from April until the end of May. Bulk inorganic N deposition from 2011 to 2014 was 4.6 kg N ha^{-1} yr^{-1} (Calvo-Fernández et al., 2017), and total N deposition ranges between 7.5 and 15 kg N ha^{-1} yr^{-1}, according to the EMEP and CHIMERE models for Spain (García-Gómez et al., 2014). Therefore, total N deposition in the study area is either lower than or within the lowest critical load value estimated to threaten the persistence of European dry *Calluna*-heathlands (i.e., 10-20 kg N ha^{-1} yr^{-1}; Bobbink et al., 2010; Hall et al., 2015). The study sites have Umbrisol soil (European Commission, 2005), characterized by sandy texture, high acidity (pH = 3.9 ± 0.14; deionized water), and low fertility. Soil depth ranges from 30 to 50 cm (on sandstone and lutite) with the following horizons: O_{if} (0-2 cm), O_h (2-5 cm), A (5-20 cm), B (20-40 cm) or A/C (20-35 cm), and C (from 30-50 cm). Therefore, soil conditions differ from the Podzol soils that are typical of many north-western European heathlands (Marcos et al., 2003). The soil of young heathland stands is covered by a shallow litter layer (<1 cm depth) distributed in discontinuous patches, while a continuous and homogeneous litter layer (2-3 cm) characterizes the mature ones. The study sites are dominated by *Calluna* (>75% cover; 20 cm height in young stands and 50 cm in mature stands), with *Erica tetralix* L. and *Vaccinium myrtillus* L. as the main accompanying species (Calvo et al., 2005). Lichens [*Cladonia* Hill ex Browne and *Cetraria islandica* (L.) Ach.] cover ca. 15% and different bryophyte species ca. 10%. The bud burst of *Calluna* plants happens in June, and the vegetation growing season period is from June to October.

3.2.2. Experimental design

In each study site, we selected two heathland areas of different ages: (1) young stands rejuvenated by prescribed fire in 2005, i.e., 8 years old at the beginning of the experiment, and (2) mature stands showing the first signs of degeneration after 30-40 years of land use abandonment (i.e., building- and mature-phase; Gimingham, 1972). We established a total of 90 2 m x 2 m plots and performed a manipulative experiment consisting of five different N fertilization treatments in addition to background atmospheric N deposition (i.e., 3 replicated plots per N treatment, age class, and site): 0 kg N ha^{-1} yr^{-1} (N0; control), 10 kg N ha^{-1} yr^{-1} (N10; low N load), 20 kg N ha^{-1} yr^{-1} (N20; medium load), and 50 kg N ha^{-1} yr^{-1} (N50; high load) of solid granules of ammonium nitrate (Fertiberia S.A.; 27% NH_4NO_3 purity) monthly added by hand to the soil surface in June-November from 2013 to 2015; and 56 kg N ha^{-1} yr^{-1} (N56; chronic high N load) added monthly in May-October from 2005 to 2015 equivalent to the highest predicted N input by 2050 for southern Europe (Galloway et al., 2004) [i.e., corresponding to ca. two times the maximum total N deposition levels in the study area at the beginning of the experiment: Rivero Fernández et al. (1996); and to ca. four times the current maximum total N deposition levels in the study area: García-Gómez et al. (2014)]. Solid granules release ammonium and nitrate slowly over the soil surface, and have been used in previous studies on heathlands responses to N fertilization (e.g., Cuesta et al., 2008; Marcos et al., 2003, 2009; Taboada et al., 2016). The young and mature plots were randomly assigned to the N treatments at each study site.

During the experiment, infestations of *Calluna* plants by the heather beetle, *Lochmaea suturalis* (Thomson, 1866), occurred in June 2008 and August 2015 at one of the three heathland sites (Riopinos II) (personal observations). The larvae of the heather beetle either partially or totally consumed the leaves of *Calluna* plants across the area where the experimental plots were located (ca. 0.5% of the heathland area was infested; only plants subjected to N fertilization were defoliated in 2008, and both untreated and fertilized plants were equally consumed in 2015) (see Taboada et al., 2016 for further information). In the two outbreak events, only the chronic high N treatment (N56) plots were affected (mature plots were severely defoliated in 2008, and both mature and young plots were marginally affected in 2015); but the consumed plants were not killed and regenerated the next growing season following the beetle's attack. After the beetle's infestation, however, the defoliated plants at the N56 mature plots were debilitated and suffered from subsequent physical damage caused by livestock trampling. We did not observe further severe damage to the vegetation due to other environmental stress factors like frost or drought.

3.2.3. Sampling methods

We collected three soil samples (topsoil, 0-5 cm below the litter layer) in each 2 m x 2 m plot in September 2014 and 2015 using a soil auger, which were combined to obtain one soil sample per plot and year. Fresh soil samples were brought to the laboratory in air-tight plastic bags, and immediately sieved (2 mm Ø) and divided in two subsamples. The first subsample was air-dried and stored in a polyethylene bag for total N, organic C, and available P analyses. We also calculated the soil C:N ratio. The second subsample was stored at -18 °C in a polyethylene bag for extractable N-NH_4^+ and N-NO_3^-, enzymatic activities, and microbial biomass C and N analyses. The thawing procedure was done at 4 °C in a refrigerator.

We randomly selected 5-10 *Calluna* plants in each plot to obtain a sufficient quantity of fine roots per plot to determine the extent of root mycorrhizal colonization in August 2015. Fine roots were manually isolated using a 400x optical microscope (M20-42149, Wild Heerbrugg, Switzerland), and subsequently stored at -18 °C until analysis.

To determine *Calluna* plant and litter N and P contents and calculate N:P ratios, we collected ten young *Calluna* apical shoots and three 5 cm x 5 cm litter layer (i.e., dead plant leaves fell to the ground) samples from each 2 m x 2 m plot in July 2014 and 2015, which were combined to obtain one shoot or litter sample per plot and year. The shoots and litter samples were dried at 40 °C for 48 h, pulverized (Pulverisette 14, Fritsch, Oberstein, Germany) and sieved (200 µm) before nutrient analyses.

3.2.4. Analytical methods

Soil total N was determined by a Kjeldhal procedure (Bremner and Mulvaney, 1982), with four reagent blanks used for each digestion batch (eight soil samples). Organic C was determined using wet oxidation with potassium dichromate (Ministerio de Agricultura, Pesca y Alimentación, 1986), and available P following the Bray-Kurtz method (Kalra and Maynard, 1991). Two reagent blanks were used for each batch of organic C determination, and one reagent blank was used for each calibration line of available P. The N-NH_4^+ and N-NO_3^- were extracted with 2M KCl (ratio 1:10 soil-extractant) according to Keeney and Nelson (1982), and measured by steam distillation with a micro-Kjeldhal automatic analyzer using the Bremner method (Bremner, 1965). Four reagent blanks were used for each batch of steam distillation (ten soil samples).

Soil acid phosphatase and β-glucosidase activities were determined colorimetrically as the amount of p-nitrophenol (*p*-NP) produced after incubation of 0.5 and 1 g of soil (37°C, 1 h) with p-nitrophenyl-phosphate and p-nitrophenyl-β-D-glucopyranoside substrates, respectively (Tabatabai and Bremner, 1969; Tabatabai, 1982). The *p*-NP formed was determined in a spectrophotometer at 400 nm (UV-1700 PharmaSpec, Shimadzu, Kyoto, Japan). Urease activity was determined following Kandeler and Gerber (1988) as the amount of N-NH_4^+ released from 1 g of soil after incubation (37 °C, 2 h) with urea substrate. The N-NH_4^+ released was measured colorimetrically at 690 nm. One sample blank for each soil sample was used in the determination of the acid phosphatase and urease activities. Two replicated measures and two sample blanks for each soil sample were used for the β-glucosidase determination. Besides, one reagent blank was used to build up each calibration line for colorimetric analyses. For β-glucosidase activity, one calibration line was built up for each soil sample using *p*-NP standard solutions incubated together with the soil.

Soil microbial biomass C and N contents were determined by the Fumigation-Extraction method (Vance et al., 1987). Estimation of soil microbial biomass N was performed by titration of total extracted N according to Brookes et al. (1985), using a K_{EN} factor of 0.45. Estimation of soil microbial biomass C was performed by wet digestion according to Vance et al. (1987), using a K_{EC} factor of 0.38. Four and two reagent blanks were used for each analysis batch of soil microbial biomass N (eight soil samples) and C (ten soil samples), respectively.

The amount of ericoid mycorrhizal colonization in *Calluna* roots was quantified in 0.5 g of the finest roots. The method consisted of a first step of staining roots with a solution of ink-vinegar (5%) according to Vierheilig et al. (1998), using Sheaffer black ink. The second step was the measurement of the percentage of roots colonized by arbuscular mycorrhizae using the intersection method by McGonigle et al. (1990), with a 400x optical microscope (M20-42149, Wild Heerbrugg, Switzerland).

Calluna shoot and litter N contents were determined by the Kjeldahl digestion method (BÜCHI Digestion Unit K-435, Flawil, Switzerland) coupled to a tritator (Metrohm 719 S tritino, Herisau, Switzerland). *Calluna* shoot and litter P contents were determined by digestion with HNO_3 (65%) and heating at 550 °C, and measured with ICP-OES (Optima 2000 DV, Perkin Elmer). Four reagent blanks were used for each digestion batch of shoot and litter N contents (eight shoot/litter samples), and one reagent blank was used for each calibration line in the colorimetric determination of shoot and litter P contents.

3.2.5. Data analyses

We fitted linear mixed models (LMMs) with a repeated measures design to test the effects of N fertilization on soil properties (nutrient contents, enzymatic activities, and microbial biomass), *Calluna* plants and litter (nutrient contents), mediated by time and the age of heathland vegetation. The response variables in the models were: (1) soil total N, (2) soil organic C, (3) soil C:N ratio, (4) soil available P, (5) soil extractable N-NH_4^+, (6) soil extractable N-NO_3^-, (7) acid phosphatase activity, (8) urease activity, (9) β-glucosidase activity, (10) soil microbial biomass C, (11) soil microbial biomass N, (12) *Calluna* shoot N content, (13) *Calluna* shoot P content, (14) *Calluna* shoot N:P ratio, (15) litter N content, (16) litter P content, and (17) litter N:P ratio. We modelled the response variables assuming a Gaussian error distribution, using the identity link function. The predictor variables (fixed factors) were age of *Calluna* plants (young and mature), the N treatment (N0, N10, N20, N50, and N56), and their interaction. The interaction term was retained in the models only when significant. Statistical significance was considered when $p < 0.05$; and significance levels of the difference between each N fertilization treatment (N10, N20, N50, and N56) and the control treatment (N0) obtained directly from the model summary outputs are indicated on the figures. The identity of the heathland sites and the sampling years were included in the models as random factors.

The normality and homogeneity of the model residuals were checked using diagnostic plots. We obtained predicted values of the response variables from the models for each heathland age and N treatment, without taking the uncertainty of the random effects parameters into account, and computed 95% confidence intervals based on a normal approximation.

We also evaluated the effects of N fertilization on root mycorrhizal colonization by performing a generalized linear model (GLM) with the percentage of *Calluna* roots colonized by mycorrhizal arbuscules as the response variable, and the age of *Calluna* plants (young and mature), the N treatment (N0, N10, N20, N50, and N56), and their interaction as the predictor variables. The interaction term was retained in the model only if significant. Statistical significance was considered when $p < 0.05$; and significance levels of the difference between each N fertilization treatment (N10, N20, N50, and N56) and the control treatment (N0) obtained directly from the model summary outputs are indicated on the figure. We modelled the response variable following a quasi-Poisson error distribution to account for overdispersion, using the log link function. We obtained model predicted values of the percentage of roots colonized by mycorrhizal arbuscules for each heathland age and N treatment.

All data analyses were carried out with R software, version 3.3.1 (R Core Team, 2016) using the ‘stats’, ‘lme4’ (Bates et al., 2015) and ‘lmerTest’ (Kuznetsova et al., 2016) packages.

3.3. Results

3.3.1. *Soil nutrient contents*

Soil extractable N-NH_4^+ content significantly increased in response to the addition of N, but only in the N56 treatment (Fig. 3.1; Table 3.1). However, there were no significant differences for soil extractable N-NO_3^- (Fig. 3.1; Table 3.1). Soil N-NH_4^+ content was ca. 10-15-fold higher than N-NO_3^- in each N treatment. Besides, significantly higher N-NO_3^- contents were observed in young heathlands than in mature ones. No changes were detected for soil available P after the addition of N, while significantly higher available P values were recorded in young heathlands, compared to mature ones (Fig. 3.1; Table 3.1). No significant differences were found for soil total N, organic C, or C:N ratio with regard to the N treatments and heathland ages (Table 3.1).

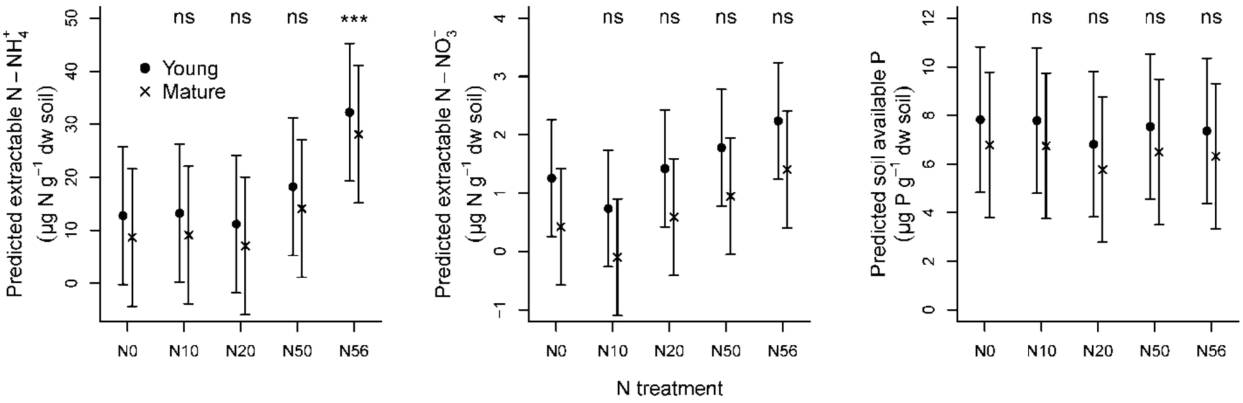

Figure 3.1. Model predicted values (mean ± 95% confidence intervals) of soil nutrient content variables in relation to stand age (young vs. mature) and the five N treatments (N0, N10, N20, N50, and N56): extractable N-NH_4^+ (µg N g^{-1} dw soil), extractable N-NO_3^- (µg N g^{-1} dw soil), and available P (µg P g^{-1} dw soil). Significance levels of the difference between each N fertilization treatment (N10, N20, N50, and N56) and the control treatment (N0) are indicated by *** ($0.001 > p$), ** ($0.01 > p > 0.001$), * ($0.05 > p > 0.01$), and ns ($p > 0.05$).

Table 3.1. LMM results ['anova()' outputs] for the effects of heathland age (young vs mature) and N treatment (N0, N10, N20, N50, and N56) on soil nutrient contents, soil enzymatic activities and soil microbial biomass nutrient contents. Standard deviations (SD) and variance components (%) of the random effects (identity of the heathland sites and the sampling years) are given. The interaction term (age x N treatment) was retained in the models only when significant. Df= degrees of freedom. Significant p-values are in bold face.

	Fixed effects				Random effects		
Response variable	Predictor variable	Df	F value	p value	Variable	SD	Variance
Soil nutrient contents							
Total N	Age	1	0.02	0.887	Site	0.103	34.90
	N treatment	4	2.01	0.095	Year	0.035	11.97
					Residual	0.157	53.13
Organic C	Age	1	0.03	0.855	Site	0.307	9.65
	N treatment	4	1.56	0.186	Year	0.237	7.45
					Residual	2.633	82.90
C:N ratio	Age	1	1.55	0.215	Site	5.741	55.70
	N treatment	4	0.58	0.677	Year	0.000	0.00
					Residual	4.566	44.30
Extractable $N\text{-}NH_4^+$	Age	1	2.97	0.087	Site	3.017	11.22
	N treatment	4	10.46	**<0.001**	Year	7.823	29.10
					Residual	16.047	59.68
Extractable $N\text{-}NO_3^-$	Age	1	4.17	**0.043**	Site	8.5 x 10^{-8}	0.00
	N treatment	4	1.53	0.196	Year	0.000	0.00
					Residual	2.737	100.00
Available P	Age	1	5.08	**0.026**	Site	2.102	34.23
	N treatment	4	0.64	0.636	Year	0.946	15.40
					Residual	3.094	50.37
Soil enzymatic activities							
Acid phosphatase	Age	1	2.98	0.086	Site	0.387	6.26
	N treatment	4	3.85	**0.005**	Year	1.454	23.54
					Residual	4.335	70.20
Urease	Age	1	0.59	0.444	Site	0.653	12.41
	N treatment	4	1.59	0.178	Year	0.946	17.99
					Residual	3.661	69.60
β-glucosidase	Age	1	0.86	0.354	Site	0.281	6.96
	N treatment	4	0.73	0.572	Year	1.116	27.59
					Residual	2.646	65.45
Soil microbial biomass							
Microbial biomass C	Age	1	3.15	0.078	Site	319.700	24.38
	N treatment	4	1.75	0.141	Year	279.500	21.31
					Residual	712.200	54.31
Microbial biomass N	Age	1	9.61	**0.002**	Site	19.650	16.80
	N treatment	4	1.29	0.275	Year	30.070	25.71
					Residual	67.24	57.49

3.3.2. Soil enzymatic activities

Acid phosphatase enzymatic activity significantly increased in response to N addition, particularly in the N56 treatment, and to a lesser extent in the N10 treatment (Fig. 3.2; Table 3.1). However, there were no significant differences in acid phosphatase activity related to heathland age. We found significant differences in urease enzymatic activity after the addition of N in the N10 treatment (Fig. 3.2; Table 3.1); but no significant differences in relation to heathland age. β-glucosidase enzymatic activity did not show significant changes in relation to N addition and heathland age (Fig. 3.2; Table 3.1).

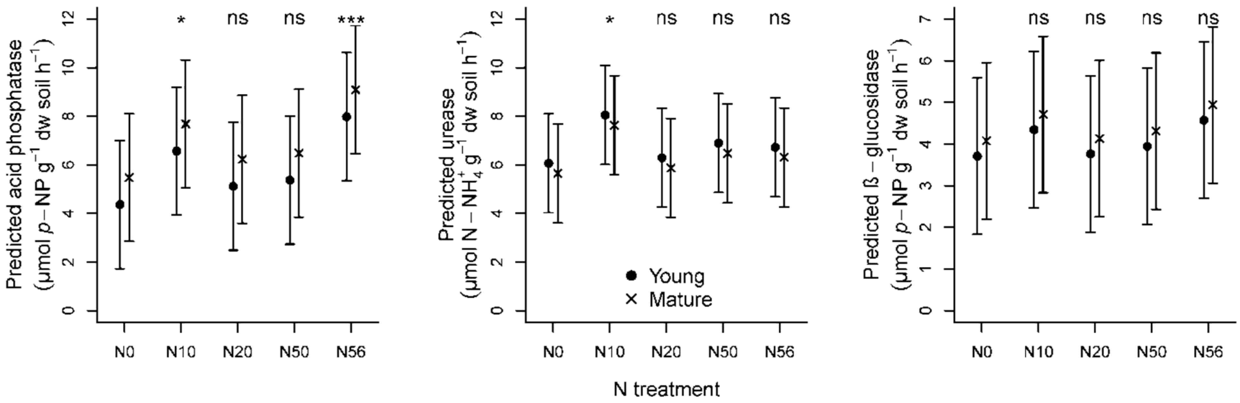

Figure 3.2. Model predicted values (mean ± 95% confidence intervals) of soil enzymatic activities in relation to stand age and the N treatments: acid phosphatase (µmol *p*-NP g^{-1} dw soil h^{-1}), urease (µmol N-NH_4^+ g^{-1} dw soil h^{-1}), and β-glucosidase (µmol *p*-NP g^{-1} dw soil h^{-1}). Significance levels of the difference between each N fertilization treatment (N10, N20, N50, and N56) and the control treatment (N0) are indicated by *** ($0.001 > p$), ** ($0.01 > p > 0.001$), * ($0.05 > p > 0.01$), and ns ($p > 0.05$).

3.3.3. Soil microbial biomass

Soil microbial biomass C and N contents did not show any significant changes in response to the addition of N (Fig. 3.3; Table 3.1). Microbial biomass N content was, however, significantly higher in young heathlands than in mature ones.

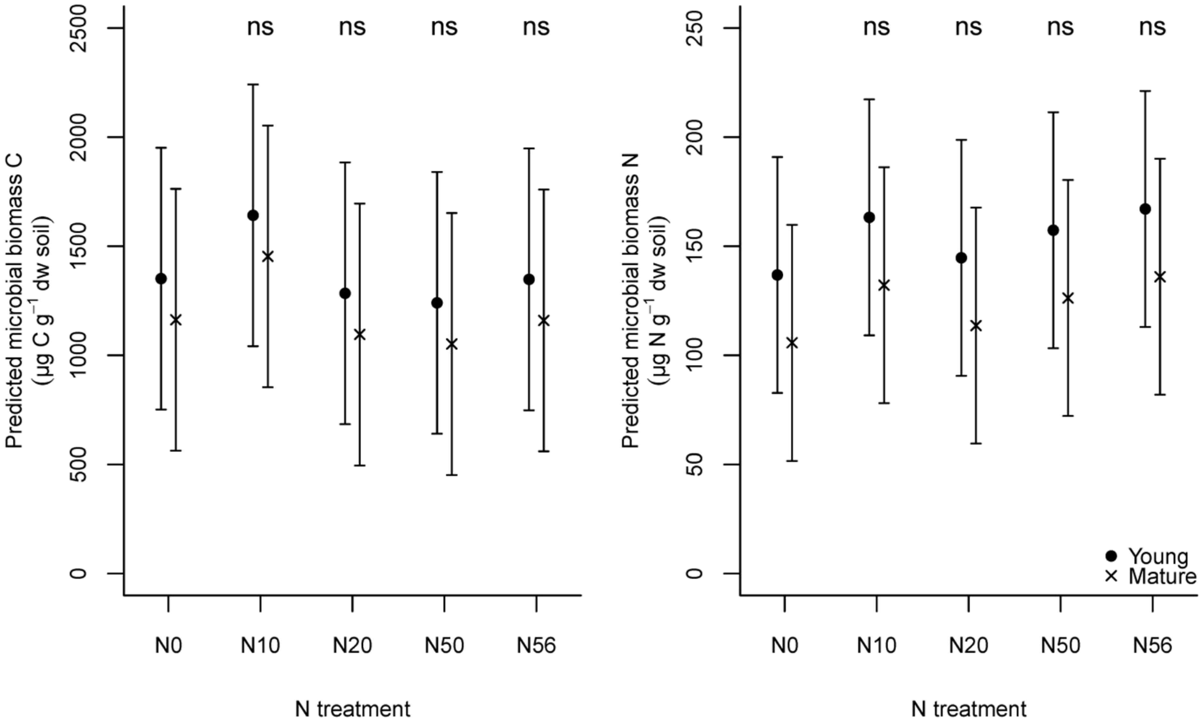

Figure 3.3. Model predicted values (mean ± 95% confidence intervals) of the nutrient contents of soil microbial biomass in relation to stand age and the N treatments: C (µg C g^{-1} dw soil), and N (µg N g^{-1} dw soil). Significance levels of the difference between each N fertilization treatment (N10, N20, N50, and N56) and the control treatment (N0) are indicated by *** ($0.001 > p$), ** ($0.01 > p > 0.001$), * ($0.05 > p > 0.01$), and ns ($p > 0.05$).

3.3.4. Calluna root mycorrhizal colonization

The percentage of *Calluna* roots colonized by mycorrhizal arbuscules was significantly higher in the N56 treatment (Fig. 3.4; Table 3.2). Moreover, the responses of mycorrhizal colonization to the N treatments were age-related, shown by the significant 'age x N treatment' interaction, with a higher percentage of colonized roots in young heathlands for the control (N0), N10 and N20 treatments, and in mature ones for the N50 and N56 treatments.

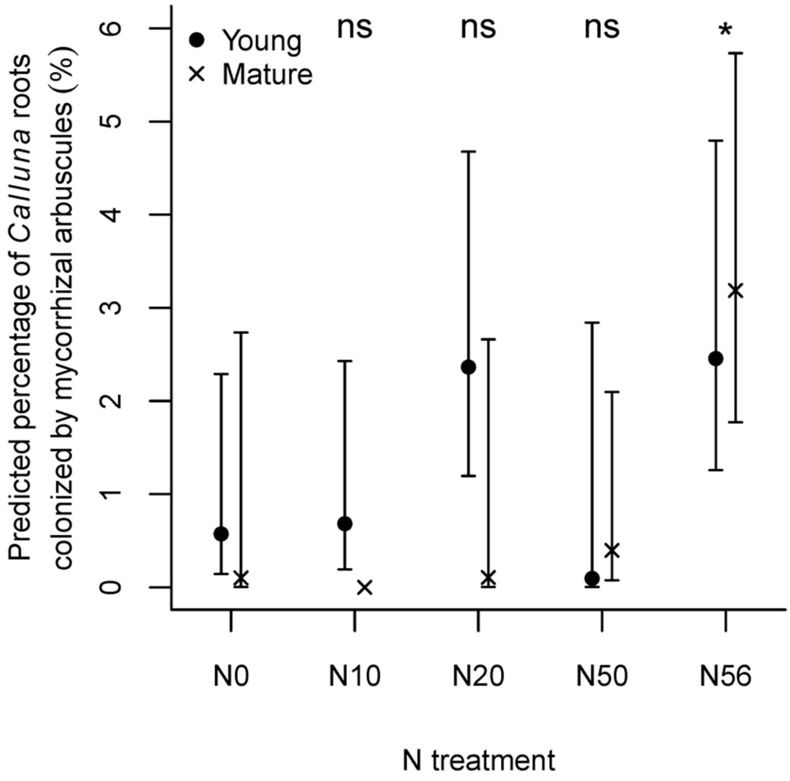

Figure 3.4. Model predicted values (mean ± 95% confidence intervals) of *Calluna* mycorrhizal colonization (% roots colonized by mycorrhizal arbuscules) in relation to stand age and the N treatments. Significance levels of the difference between each N fertilization treatment (N10, N20, N50, and N56) and the control treatment (N0) are indicated by *** ($0.001 > p$), ** ($0.01 > p > 0.001$), * ($0.05 > p > 0.01$), and ns ($p > 0.05$).

Table 3.2. GLM results [‘anova()’ output] for the effects of heathland age (young, mature) and N treatment (N0, N10, N20, N50, and N56) on *Calluna* mycorrhizal colonization. Df= degrees of freedom. Significant p-values are in bold face.

Response variable	Predictor variable	Df	Deviance	Residual deviance	p value
Mycorrhizal colonization *Calluna* roots colonized by mycorrhizal arbuscules	NULL			284.55	
	Age	1	5.18	279.37	0.156
	N treatment	4	76.57	202.80	**<0.001**
	Age:N treatment	4	32.24	170.57	**0.014**

3.3.5. Calluna shoot and litter nutrient contents

Both *Calluna* shoot N and P contents significantly increased as a result of N fertilization, particularly under the N56 treatment (Fig. 3.5; Table 3.3). Also, young *Calluna* plants had significantly higher shoot N and P contents than mature ones.

Litter N content significantly increased in the N50 and N56 treatments (Fig. 3.5; Table 3.3). Besides, higher litter N content was found in young heathlands than in mature ones. A significant ‘age x N treatment’ interaction was found for litter P content (Fig. 3.5; Table 3.3), with higher values in young heathlands for the N20, N50 and N56 treatments, and in mature ones for the control treatment (N0).

Shoot and litter N:P ratios significantly increased after N addition, and achieved maximum values in the N50 and N56 treatments, respectively, for both heathland ages (Fig. 3.5; Table 3.3).

Table 3.3. LMM results [‘anova()’ outputs] for the effects of heathland age (young vs mature) and N treatment (N0, N10, N20, N50, and N56) on *Calluna* shoot and litter nutrient contents. Standard deviations (SD) and variance components (%) of the random effects (identity of the heathland sites and the sampling years) are given. The interaction term (age x N treatment) was retained in the models only when significant. Df= degrees of freedom. Significant p-values are in bold face.

	Fixed effects				Random effects		
Response variable	Predictor variable	Df	F value	p value	Variable	SD	Variance
Calluna shoot nutrient contents							
N content	Age	1	22.35	**<0.001**	Site	0.023	13.96
	N treatment	4	58.97	**<0.001**	Year	0.041	24.56
					Residual	0.102	61.48
P content	Age	1	11.86	**<0.001**	Site	0.011	39.98
	N treatment	4	8.42	**<0.001**	Year	0.004	14.62
					Residual	0.013	45.40
N:P ratio	Age	1	2.51	0.115	Site	2.033	40.53
	N treatment	4	6.28	**<0.001**	Year	0.582	11.61
					Residual	2.400	47.86
Litter nutrient contents							
N content	Age	1	21.50	**<0.001**	Site	0.084	24.55
	N treatment	4	39.12	**<0.001**	Year	0.104	30.43
					Residual	0.154	45.03
P content	Age	1	5.96	**0.016**	Site	0.010	33.54
	N treatment	4	3.18	**0.015**	Year	0.008	25.56
	Age:N treatment	4	3.75	**0.006**	Residual	0.013	40.90
N:P ratio	Age	1	0.01	0.914	Site	3.002	30.60
	N treatment	4	4.01	**0.004**	Year	1.460	14.88
					Residual	5.348	54.52

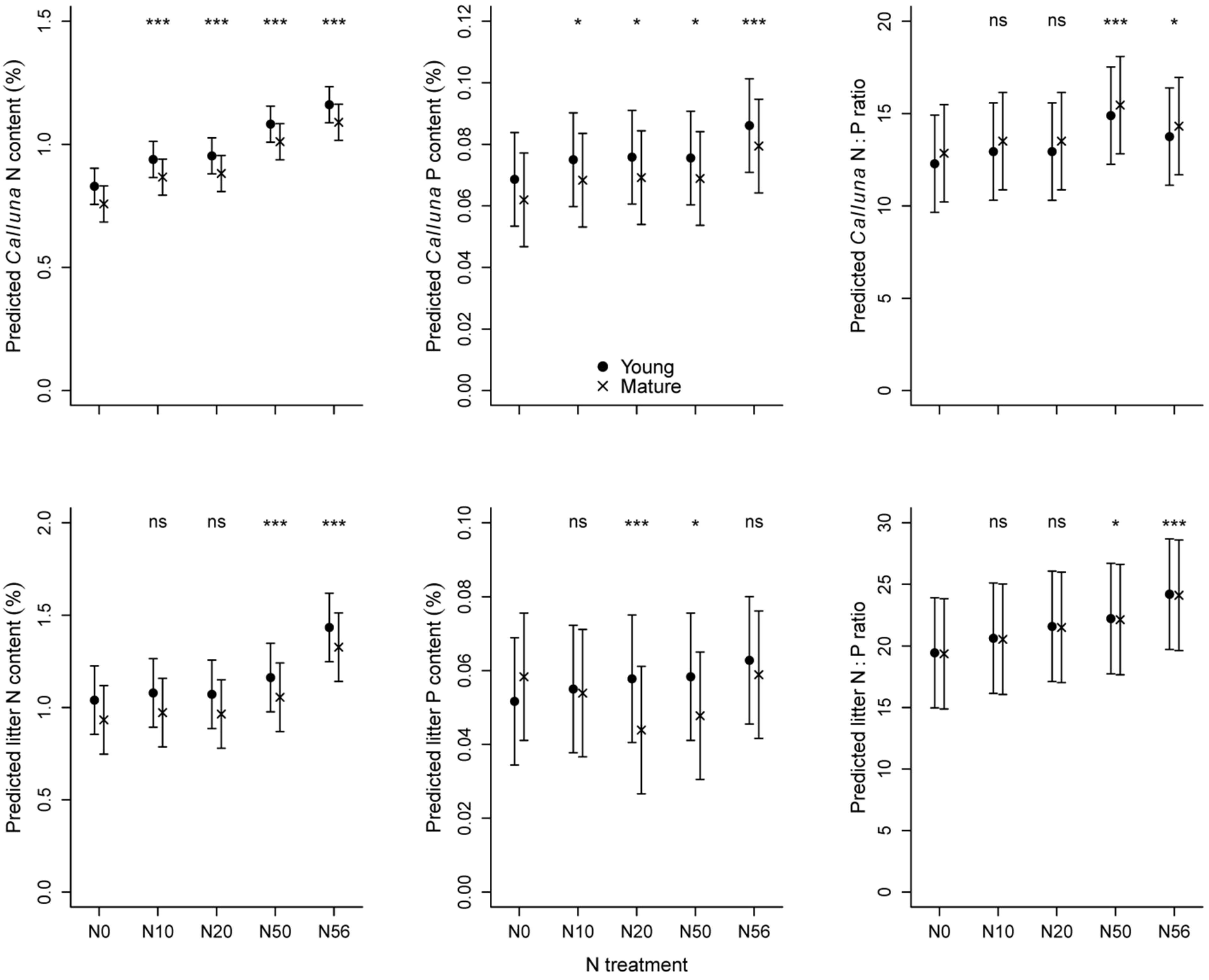

Figure 3.5. Model predicted values (mean ± 95% confidence intervals) of *Calluna* shoot and litter nutrient contents in relation to stand age and the N treatments: N (%), P (%), and N:P ratio. Significance levels of the difference between each N fertilization treatment (N10, N20, N50, and N56) and the control treatment (N0) are indicated by *** (0.001 > p), ** (0.01 > p > 0.001), * (0.05 > p > 0.01), and ns (p > 0.05).

3.4. Discussion

3.4.1. Time-related effects of N fertilization

Several previous field-scale surveys and N-fertilization experiments carried out in north-western European heathlands provided strong evidence of the impact of N deposition on soil nutrients, indicated by an increase in extractable N-NH_4^+ and N-NO_3^- under high-N inputs (e.g., Phoenix et al., 2012; Southon et al., 2013). Similar results were also reported from other systems such as subalpine forests (Boot et al., 2016), permafrost peatlands (Song et al., 2017), and semiarid Mediterranean shrublands (Ochoa-Hueso et al., 2013, 2014). In Cantabrian marginal montane heathlands, we only observed an increase in soil extractable N-NH_4^+ in the chronic high N treatment (N56; 10 years), but no changes in soil extractable N-NO_3^-. These results suggest that in these montane heathlands a shift in soil extractable N is only to be expected under high N loads. This coincides with findings from upland and lowland heaths, in which significant responses of soil N-NO_3^- and N-NH_4^+ were mainly observed at the highest N deposition rates (up to 120 kg N ha^{-1} yr^{-1}; Phoenix et al., 2012). Moreover, soil extractable N-NH_4^+ contents were about 10 to 15-fold higher than N-NO_3^- in all the montane heathland stands, and particularly in the chronic high N treatment (N56), as was also reported by Boot et al. (2016) in a subalpine forest ecosystem under long-term (17-year) N load. It is very likely that the unique environmental conditions [low soil pH values (3.9), low winter temperatures and summer droughts] of the heathlands studied had inhibited nitrification [net nitrification rates around 0.25-1.5 g N m^{-2} $month^{-1}$ (unpublished data)] (Stevens et al., 2011), resulting in a notable accumulation of soil extractable N-NH_4^+ (Nielsen et al., 2009; Stevens et al., 2011). On the other hand, soil organic C, total N, and available P were not affected by N fertilization, probably due to the slow rate of change in the C and N pools in response to increased N availability (Ochoa-Hueso et al., 2013, 2014). Therefore, it might take more than 10 years of N fertilization to alter the soil C and N pools in montane *Calluna*-heathlands due to the short period of microbial physiological activity, since chronic high N loads might result in the production of N-rich litter that would be very slowly decomposed by soil microbes and incorporated into the topsoil stocks of C (de Vries et al., 2009) and N (Pilkington et al., 2005b).

The observed N-driven changes in soil nutrient contents may also be related to the alteration in the functioning of soil microorganisms and the resulting soil extracellular enzymatic activities, determined by the levels of metabolic nutrient demands (Ochoa-Hueso et al., 2011; Sinsabaugh and Follstad, 2012; Jian et al., 2016; Song et al., 2017). We reported a significant rise in the activity of the acid phosphatase enzyme in response to N fertilization, especially in the chronic high N treatment (N56). Johnson et al. (1998) and Pilkington et al. (2005a) also found that the greatest soil acid phosphatase activity corresponded to their highest long-term N-addition treatment (120 kg N ha^{-1} yr^{-1}) in upland heathlands; whereas a N deposition load of only 10 kg N ha^{-1} yr^{-1} was sufficient to increase the acid phosphatase activity in a low-alpine heathland (Papanikolaou et al., 2010). The observed increase in soil acid phosphatase activity in our studied montane heathlands could be explained by changes in soil nutrients, resulting in P deficiency for plants and soil microbes (Phoenix et al., 2003; Pilkington et al., 2005a; Ochoa-Hueso et al., 2014). This nutritional imbalance could be compensated for by the microbial and plant excretion of phosphatase enzymes required for the mineralization of organic P (Pilkington et al., 2005a; Rowe et al., 2008; López-Poma and Bautista, 2014); since phosphatase activity can be modulated through enzyme excretion or activity inhibition depending on soil P availability (Johnson et al., 1999; Phoenix et al., 2003; López-Poma and Bautista, 2014).

Soil urease activity, however, did not change in response to N fertilization, as high soil inorganic N availability inhibited this enzymatic activity through a reduced synthesis and release of urease enzyme by soil microbes (Ajwa et al., 1999; Sinsabaugh and

Follstad, 2012; Fatemi et al., 2016; Song et al., 2017). Likewise, we did not find a significant response of β-glucosidase enzyme activity to N fertilization, similarly to the findings from low-alpine heathlands (Papanikolaou et al., 2010), forests (Boot et al., 2016; Fatemi et al., 2016), and peatlands (Song et al., 2017). This lack of response is most likely the result of (1) the unaltered soil organic C contents, suggesting that the activity of this enzyme is substrate-dependent (López-Poma and Bautista, 2014; Cenini et al., 2016), and (2) of the fact that soil microbial communities, which predominantly synthetize β-glucosidase enzyme, are not limited by soil C availability (Sinsabaugh and Follstad, 2012). Indeed, we found no significant changes in soil microbial biomass C and N in response to increased N availability, in agreement with other studies from temperate (Nielsen et al., 2009) and arctic (< 10 years of N inputs; Rinnan et al., 2007) heathlands; but different from previous studies in which both or either soil microbial biomass N or C increased (e.g., upland and lowland heathlands: Johnson et al., 1998; Power et al., 2006; temperate forests: Du et al., 2014) or decreased (e.g., peatlands: Song et al., 2017; forests: Boot et al., 2016). Our results support the fact that there is a reduced microbial acquisition and immobilization of nutrients that maintains the low soil fertility status of montane heathlands (Nielsen et al., 2009). Furthermore, the distinctive climatic conditions of our montane study area, characterized by low temperatures, prolonged snow cover until late spring, and a brief summer drought (Calvo-Fernández et al., 2017), could have also influenced the low rates of soil microbial nutrient acquisition (Hagedorn et al., 2010; Calvo-Fernández et al., 2015). Moreover, previous studies indicated that longer-term (> 10-year) N inputs may be required for producing significant shifts in the nutrient content of the soil microbial biomass in heathlands due to the slow organic matter decomposition rates (Rinnan et al., 2007; Contosta et al., 2015).

As we hypothesized, N addition increased *Calluna* tissue N and P contents, especially in the chronic high N treatment (N56), and *Calluna* N:P ratio, particularly in the high (N50) and chronic high (N56) N treatments. This is in agreement with previous N-fertilization experiments and field-scale surveys performed in north-western European heathlands (Pilkington et al., 2005b; Jones and Power, 2012; Southon et al., 2012). According to the N:P threshold values proposed by Güsewell (2004) in terrestrial plant communities (i.e., N:P ratio <10 for N-limited and >20 for P-limited systems), our results indicated that montane *Calluna* heathlands subjected to long-term N fertilization may not be limited by either N or P (see Britton et al., 2008; von Oheimb et al., 2010; Friedrich et al., 2011). The reported increase in *Calluna* tissue N and P contents may be related to the observed increases in litter N and P contents (Jones and Power, 2012), likely due to the inputs of the N and P enriched shoots to the litter layer (Pilkington et al., 2005b). However, the increase in *Calluna* tissue and litter P contents did not alter soil available P content, since all newly-mineralised soil P may have been immediately uptaken by the plants or incorporated into the soil microbial biomass to satisfy their enhanced P demands in response to the addition of N (Johnson et al., 1998, 1999; Rowe et al., 2008; Friedrich et al., 2011; Jones and Power, 2015). Particularly, the enhanced P demand of *Calluna* plants in the chronic high N treatment (N56) was probably satisfied by: (1) the observed increase in the activity of soil acid phosphatase enzyme that is necessary for the mineralization of soil organic P (Pilkington et al., 2005a; Rowe et al., 2008; López-Poma and Bautista, 2014); and by (2) the observed increase in the degree of *Calluna* root mycorrhizal colonization that might enhance plant nutrient uptake (Díaz et al., 2006; Rowe et al., 2008; Jones and Power, 2012). Nonetheless, a decrease in root mycorrhizal colonization with increasing N inputs was observed by Camenzind et al. (2016) in an N-fertilization experiment in montane forests, pointing out that the response of mycorrhizal fungi to N load depends on the previous soil fertility status.

3.4.2. Age-related effects of N fertilization

As expected, greater above-ground biomass in mature heathlands required higher N and P amounts to increase the relative nutrient contents of *Calluna* plants, and this effect was amplified by N fertilization. As a result, lower soil nutrient availability, mainly extractable N-NO_3^- and available P, was found in mature stands as compared to young ones. Furthermore, higher N demand by mature *Calluna* plants may have induced a lower N content in the soil microbial biomass as *Calluna* plants are better competitors than microbes for soil nutrients (Harrison et al., 2008). Similarly, higher P demand by mature *Calluna* plants may have induced a lower litter P content in the medium (N20), high (N50) and chronic high (N56) N treatments compared to young ones, likely due to P resorption from senescing plant biomass to physiologically-active shoots (Aerts and Chapin, 2000).

Mycorrhizal colonization of *Calluna* roots varied not only with plant age (Read and Pérez-Moreno, 2003), but also with the amount of experimental N-load. Young *Calluna* plants in the control (N0), low (N10) and medium (N20) N treatments had significantly higher percentages of roots colonized by ericoid mycorrhizal fungi than mature plants (see Johansson, 1994), possibly to facilitate nutrient mobilization (Read and Pérez Moreno, 2003; Díaz et al., 2006). However, mature *Calluna* plants subjected to the high (N50) and chronic high (N56) N treatments showed significantly greater mycorrhizal root colonization, probably in response to their higher P demands (Johnson et al., 1999;

Díaz et al., 2006). Besides, higher aboveground biomass of mature stands could transfer greater amounts of photosynthesized-carbohydrates to arbuscular mycorrhizal fungi for extending the mycelial network to access to soil nutrients (Johnson et al., 2005).

Finally, young *Calluna* plants had significantly higher shoot N and P and litter N contents than mature ones across all N treatments, very likely indicating that (1) young plants may acquire high amounts of nutrients to support their greater and faster annual growth rates (Giminghan, 1972; Jones and Power, 2015), while (2) mature plants with lower annual growth rates may store the acquired nutrients more evenly in their higher above- and below-ground plant biomass.

3.4.3. Implications for ecosystem sustainability

Our findings demonstrated that many components of the soil-microbial-enzyme system of marginal montane heathlands did not respond to the experimental addition of N, even after long-term (10-year) high N load (56 kg N ha^{-1} yr^{-1} plus background N deposition). This might suggest that montane heathland ecosystems may either be quite resistant and adapt well to enhanced N availability (Marcos et al., 2003; Calvo et al., 2005) or require longer time periods of N inputs before the deleterious effects of N loads on their biogeochemical properties become evident. It seems rather likely that the limited biotic activity and the slow nutrient cycling associated to the particular climatic conditions of montane heathlands may be causative factors for this lack of response to the added N (Hagedorn et al., 2010). Furthermore, together with the apparent ability of montane *Calluna* plants to withstand disturbance factors (i.e., insect defoliation, drought and frost; see '3.2.2. Experimental design'), suggests that these marginal heathlands may be quite resilient to N load. Calvo et al. (2007) also found that the vegetation of Cantabrian montane heathlands is resilient to disturbances like N loading and intense management practices, recovering the pre-disturbed vegetation structure and composition. However, further research is needed to fully understand the effects of N deposition and its interactions with other global environmental change factors (Meyer-Grünefeldt et al., 2016).

3.5. Conclusions

The results found in our study constitute a novelty in the field of heathland ecology in the context of accelerating global environmental change. This is the first assessment of the impact of cumulative N loads on the plant-soil-microbial-enzyme system of heathlands at their southern distribution limit, in relation to the life-history stage of the dominant dwarf-shrub. Our results demonstrated for the first time that many biogeochemical properties of marginal montane heathlands (including soil organic C and total N, extractable N-NO_3^- and available P; microbial biomass nutrient contents; and urease and β-glucosidase enzyme activities) do not respond to the enhanced availability of N. However, N fertilization leads to increased soil extractable N-NH_4^+, enhanced *Calluna* tissue N and P contents, increased litter N content, and enhanced shoot and litter N:P ratios; these effects being amplified under chronic (10-year) high N inputs (56 kg N ha^{-1} yr^{-1} plus background N deposition). N enrichment further results in a greater P demand by *Calluna* plants, which is supplied by (1) an increase in acid phosphatase enzyme activity and by (2) higher percentages of root mycorrhizal colonization by ericoid fungi.

Furthermore, our study highlights the relevance of taking into account the age of vegetation when investigating the responses of the plant-soil-microbial-enzyme system of European heathlands to cumulative N loads. *Calluna* stands in the mature phase of development have lower soil extractable N-NO_3^- and available P, and lower plant tissue N and P contents and litter N content than young ones, owing to higher nutrient demands and uptake rates by mature *Calluna* plants with more above-ground biomass. These greater nutrient demands of mature *Calluna* plants possibly lead to (1) lower N content in the soil microbial biomass and (2) greater root mycorrhizal colonization by ericoid fungi under high N availability.

3.6. References

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CHAPTER 4

Response of montane heathland vegetation to nitrogen fertilization depending on time-scale and loading

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Abstract

Increased atmospheric nitrogen (N) loads have the potential to alter plant community composition and structure in N-limited ecosystems such as *Calluna vulgaris* L. (Hull) heathlands. We investigated the effects of five N treatments (0, 10, 20 and 50 kg N ha^{-1} yr^{-1} for 2 years; and 56 kg N ha^{-1} yr^{-1} for 10 years) on plant community composition, species richness, life-form cover, and vital rates of *Calluna vulgaris*. The study was conducted in marginal montane *Calluna*-heathlands at two different life-cycle stages (young- and mature-phase). Our findings revealed that *Calluna* vital rates (i.e., current year's shoot growth and flowering) were stimulated with increasing N loads, although shifts to decreasing trends were observed under chronic N loads (10 years) in young stands. There were no significant N-related changes in plant species richness and community composition. However, there was a significant cover increase in graminoids and forbs (both annual and perennial) in response to enhanced N loads. Non-vascular species such as bryophytes and lichens showed strong cover decline under chronic N inputs. These results highlighted the impact of accumulated high N load on the heathland vegetation structure. Young stands displayed longer *Calluna* shoots than mature stands due to higher plant productivity rates in the building phase. Besides, young heathlands also showed different plant community composition and higher species richness, since the opened gaps in the dwarf-shrub-canopy in young stands led to colonization by graminoid and forb species. In order to preserve the biodiversity of montane heathland ecosystems, we proposed traditional management cycles by prescribed burning (20-30 years) to achieve rejuvenated vegetation stands.

4.1. Introduction

Human activities have dramatically increased the mobility and deposition of reactive forms of nitrogen (Nr) in recent decades over terrestrial ecosystems (Galloway et al., 2004; Stevens et al., 2011). This anthropogenic disruption of the global N cycle is responsible for biodiversity loss in a wide variety of terrestrial ecosystems (Sala et al., 2000; Bobbink et al., 2010; Field et al., 2014; Britton et al., 2017). Understanding the magnitude of the effects of these N depositions will be of major importance for the preservation of ecosystem biodiversity (Phoenix et al., 2012; Field et al., 2014).

Semi-natural ecosystems such as heathlands are naturally adapted to low levels of N deposition and occur in soils of low N availability (Britton et al., 2001; De Graaf et al., 2009). The effects of N deposition on this type of ecosystem mainly depend on the input rate, with a defined critical load above which significant harmful effects are expected on the ecosystem (10-20 kg N ha^{-1} yr^{-1} for dry heathlands; Bobbink et al., 2010). However, time-related consequences of N inputs should also be considered in the assessment of N deposition effects (De Schrijver et al., 2011; Humbert et al., 2016), since cumulative inputs could result in a potentiation of ecosystem responses with only low N loads (Clark and Tilman, 2008; Phoenix et al., 2012; Humbert et al., 2016); or even a shift from positive effects related to vegetation growth at the beginning of N additions to adverse changes latterly (Lee and Caporn, 1998; Southon et al., 2012).

Commonly, increased N deposition in heathlands, and particularly long-term inputs as occur in central and north-western Europe, will result in a reduction in plant diversity through progressive changes in species composition and a broad loss of N-sensitive species (Van den Berg et al., 2005; De Graaf et al., 2009; Maskell et al., 2010; Stevens et al., 2011; Southon et al., 2013; Field et al., 2014; Britton et al., 2017). It is well documented that enhanced available N causes competitive exclusion of the dominant dwarf-shrub species, such as *Calluna vulgaris* (L.) Hull (henceforth referred to as *Calluna*), by relatively fast-growing nitrophilous species such as grasses (Aerts and Heil, 1993; Bobbink et al., 2010; Friedrich et al., 2011b). Several studies have demonstrated that during the first years of increased N availability there are stimulations of shoot growth and flowering of *Calluna* (Lee and Caporn, 1998; Britton and Fisher, 2008; Cuesta et al., 2008; Phoenix et al., 2012); but over time, a negative effect on *Calluna* shoot growth was found due to long-term N accumulation in the ecosystem (Carroll et al., 1999; Southon et al., 2012). Furthermore, negative effects since the first years under high N availability were found in the structure and composition of N-sensitive bryophytes and lichens as a result of ammonium toxicity and acidity conditions (Southon et al., 2013; Nielsen et al., 2014; Bähring et al., 2017). These cryptogam species were replaced by N-tolerant species such as grasses in central European heathlands under higher N loads than 20 kg N ha^{-1} yr^{-1} (Bähring et al., 2017).

Traditional management practices in heathlands (i.e., sod-cutting, mowing and prescribed burning) have enabled a rejuvenated vegetation to be maintained (Gimingham, 1972; Härdtle et al., 2006; Jones et al., 2017) and the negative effects of atmospheric N inputs mitigated by the removal of the N pool in the vegetation biomass (Härdtle et al., 2006, 2009; von Oheimb et al., 2009). To date, little is known about the influence of the *Calluna* life-cycle stage on the response to increasing N inputs (Jones and Power, 2015). It has been reported that the pioneer and building phases after the application of management practices are the most sensitive growth-phases for changes in species composition in central and north-western European heathlands (Britton et al., 2000; Friedrich et al., 2011b), since the opened canopy patches in young stands could be more easily occupied by N-tolerant grasses (Britton et al., 2000, 2001). However, there are no studies about the effects of atmospheric N depositions mediated by the *Calluna* life-cycle stage on heathlands located at their southern-most distribution limit (Cantabrian Mountains, NW Spain), where they have traditionally been managed by frequent burning practices, and therefore, the heathland vegetation is well adapted to perturbations through high resilience capacity (Calvo et al., 2002a, 2007).

In this study, we aim to evaluate the effects of different levels of N loads (0, 10, 20 and 50 kg N ha^{-1} yr^{-1} for 2 years, and 56 kg N ha^{-1} yr^{-1} for 10 years) on the vegetation structure and plant community composition in montane *Calluna*-heathlands, focusing on the impact of cumulative N load over time, and considering two different *Calluna*-heathland life-cycle stages (i.e., young/building- and mature-phase; Gimingham, 1972). We hypothesized that an increase in N loads will result in: (1) a decline in plant species richness due to the loss of N-sensitive species (Maskell et al., 2010; Caporn et al., 2014); (2) an increase in N-tolerant graminoids cover and no changes in heathland-dominant woody species cover (Alonso et al., 2001; Calvo et al., 2005; Bähring et al., 2017); (3) a decline in non-vascular plants cover related to ammonium toxicity and soil acidification (Carroll et al., 1999; Stevens et al., 2016); (4) an increase in *Calluna* shoot growth and flowering (Lee and Caporn, 1998; Cuesta et al., 2008; Southon et al., 2012). We also hypothesized that the impacts of N inputs will be greater with chronic N loads (Phoenix et al., 2012; Humbert et al., 2016), especially for the cover decline of the most N-sensitive species (Phoenix et al., 2012; Southon et al., 2012, 2013). Furthermore, we also expected that the heathland life-cycle stage (young- and mature-phase) was able to influence the vegetation response to increasing N depositions (Jones and Power, 2015;

Meyer-Grünefeldt et al., 2015), since the effects of N inputs on the vegetation structure and composition will be stronger in more dynamic young stands (higher rates of plant growth and nutrient uptake) compared to the stabilized state of mature stands (Gimingham, 1972).

4.2. Material and methods

4.2.1. Study area

The study area is located on the southern side of the Cantabrian Mountains range (NW Spain). Three homogeneous *Calluna*-heathland sites were selected, situated at least 3 km apart from each other, and a maximum of 8 km between the most distant sites: Riopinos I (1653 m a.s.l., 43°02'N, 5°24'W), Riopinos II (1567 m a.s.l., 43°02'N, 5°26'W), and San Isidro (1636 m a.s.l., 43°03'N, 5°21'W). The study area has a Eurosiberian climate, with total annual precipitation of 1308 mm and mean annual temperature of 7.0 ± 4.9 °C for the year 2014. It is also characterized by a drought period in summer between July-August, and a snow cover in winter which remains until the end of May. The growing season comprises from June to October, with the vegetative bud burst in June and the flowering bud burst in July. Bulk N deposition in the study area was 4.6 kg N ha^{-1} yr^{-1} for the period 2011-2014 (Calvo-Fernández et al., 2017), and total N deposition in 2008 ranged from 7.5 to 15 kg N ha^{-1} yr^{-1} according to the EMEP and CHIMERE models for Spain (García-Gómez et al., 2014), lower than or within the critical load range that threatens the persistence of European dry *Calluna*-heathlands (i.e., 10-20 kg N ha^{-1} yr^{-1}; Bobbink et al., 2010). Soils are Umbrisol (European Commission, 2005), developed over shales and sandstones (San Isidro) and quartzite rocks (Riopinos I and II) (Marcos et al., 2015). These soils are characterized by high acidity (pH 3.9; deionized water), low fertility, with a sandy texture and high permeability (Marcos et al., 2009).

4.2.2. Experimental design

We selected two heathland areas of different life-cycle stages in each study site: (1) young stands rejuvenated by prescribed fire in 2005, i.e., 8 years old at the beginning of the experiment (young or building-phase; Gimingham, 1972), and (2) mature stands showing the first signs of degeneration after 30-40 years of land use abandonment (mature-phase; Gimingham, 1972). We established a total of 90 2 m x 2 m plots and performed a manipulative experiment consisting of five randomly-assigned N treatments (i.e., 3 replicated plots per N treatment, age class, and site): 0 kg N ha^{-1} yr^{-1} (N0; control), 10 kg N ha^{-1} yr^{-1} (N10; low N load), 20 kg N ha^{-1} yr^{-1} (N20; medium N load), and 50 kg N ha^{-1} yr^{-1} (N50; high N load) from 2013 (short-term; 2 years); and 56 kg N ha^{-1} yr^{-1} (N56; chronic high N load) from 2005 (long-term; 10 years). Fertilization consisted of solid granules of ammonium nitrate (Fertiberia S.A.; 27% NH_4NO_3 purity) added monthly by hand to the soil surface from June to November for low (N10), medium (N20), and high N loads (N50); and from May to October for chronic high N load (N56).

4.2.3. Vegetation sampling

In each plot, the lengths of five randomly selected current year's shoots of *Calluna* were measured at the end of July 2014 to evaluate the effects on *Calluna* vital rates. Moreover, to assess the effect of N additions on the reproductive potential of *Calluna*, we counted the number of flowers per shoot (bugs, opened and faded flowers) corresponding to the five *Calluna* shoots previously selected for length measurement in each plot.

In each 2 m x 2 m plot, the percentage of cover for each vascular and non-vascular species was measured in the first year after starting experimental N treatments (July 2014). Non-vascular species with difficulties in field identification were amalgamated at the division level (Bryophyta Schimp.) and the generic level (*Cladonia* spp. P. Browne), as well as the generic level for vascular plants (*Luzula* spp. DC., and *Scilla* spp. L.).

4.2.4. Data analysis

Total plant species richness was calculated as the sum of vascular and non-vascular species number. The single cover values of each species were used to determine the total cover of the following life forms: woody, perennial forbs, perennial graminoids, annual forbs, annual graminoids, bryophytes and lichens.

Differences in plant species composition (species cover data) among N treatments and heathland ages were tested with non-metric multidimensional scaling (NMDS) and permutational multivariate analysis of variance (MANOVA) [999 permutations; sequential sums-of-squares (type I)] using community distance matrices and the ‘metaMDS()’ and ‘adonis2()’ functions. We used the Bray-Curtis dissimilarity index to calculate the site x species distance matrix, and evaluated the quality of the ordination with the stress value (i.e., the mismatch between distance measures and distances in the ordination space). Only species present in at least 20% of the sampling plots (i.e., 6 plots, 19 species) were included in the ordination. Predictors were the age of *Calluna* plants (young and mature), the N treatment (N0, N10, N20, N50, and N56), and their interaction.

We fitted linear mixed models (LMMs) to test the effects of heathland age and N fertilization on the vital rates of *Calluna* plants. The response variables in the models were current year's *Calluna* shoot length and number of flowers. We modelled the response variables assuming a Gaussian error distribution, using the identity link function. The predictor variables (fixed factors) were the age of *Calluna* plants (young and mature), the N treatment (N0, N10, N20, N50, and N56), and their interaction. Minimal adequate models (MAMs) to describe the data were determined by the Akaike's information criterion (AIC) by means of the 'anova ()' function. Also, we initially used the AIC to compare (a) the fit of LMMs with the identity of the sampling plots nested within each heathland site as a random factor, (b) the fit of LMMs with the identity of the heathland sites as a random factor, and (c) the fit of generalised least squares (GLS) models without the random factor, using restricted maximum likelihood estimation (REML) procedures, by means of the 'anova()' and 'gls()' functions. Models with the identity of the heathland sites as a random factor were the most parsimonious ones, and, therefore, the ones that are reported.

We evaluated the effects of heathland age and N fertilization on total plant species richness by performing generalized linear mixed models (GLMMs) with the total number of species as the response variable, modelled following a Poisson error distribution (log link function). We also fitted analogous GLMMs with the percentage cover of the different life forms: (1) perennial forbs, (2) annual forbs, (3) perennial graminoids, (4) annual graminoids, (5) woody species, (6) bryophytes, and (7) lichens, as the response variables modelled following a quasi-Poisson error distribution (log link function) to account for overdispersion. The predictor variables (fixed factors) were the age of *Calluna* plants, the N treatment, and their interaction. The interaction term was retained in the models only when significant. The identity of the sampling plots nested within each heathland site was included in the models as random factor and retained only when accounted for >5% of the variation.

We obtained predicted values of all the response variables in the models (except for annual forbs and annual graminoids due to very low cover values) for each *Calluna* age and N treatment, without taking the uncertainty of the random effects parameters into account, and computed 95% confidence intervals based on a normal approximation. Significance levels of the difference between each N fertilization treatment (N10, N20, N50, and N56) and the control treatment (N0) obtained directly from the model summary outputs, are indicated in the figures.

All data analyses were carried out with R software, version 3.3.1 (R Core Team, 2016) using the 'stats', 'MASS' (Venables and Ripley, 2002), 'nlme' (Pinheiro et al., 2016), and 'vegan' (Oksanen et al., 2017) packages.

4.3. Results

4.3.1. Calluna vital rates

The current year's *Calluna* shoot length displayed a significant progressive increase related to the rise in N load (Table 4.1; Fig. 4.1), being significantly higher in the N20, N50 and N56 treatments in relation to the control treatment (N0). The heathland life-cycle stage had a significant influence in *Calluna* shoot growth, displaying longer current year's shoots in young heathlands than in mature ones. There was a different response pattern to the increase in N load between both heathland ages, with significant 'age x N treatment' interaction (Table 4.1). Young heathlands showed a decreasing trend in *Calluna* shoot length under chronic high N treatment (N56), whereas mature heathlands showed a progressive increase in shoot length with increasing N loads.

There was a significant increase in the number of *Calluna* flowers per shoot in the N20, N50 and N56 treatments (Table 4.1; Fig. 4.1). The number of flowers was higher in young stands but without significant differences. However, significant differences were displayed between the heathland life-cycle stages to increasing N loads ('age x N treatment' interaction; Table 4.1). Mature heathlands showed a progressive increase in the number of *Calluna* flowers with increasing N loads, whereas the young ones displayed a decreasing trend in the chronic high N treatment (N56) (Fig. 4.1).

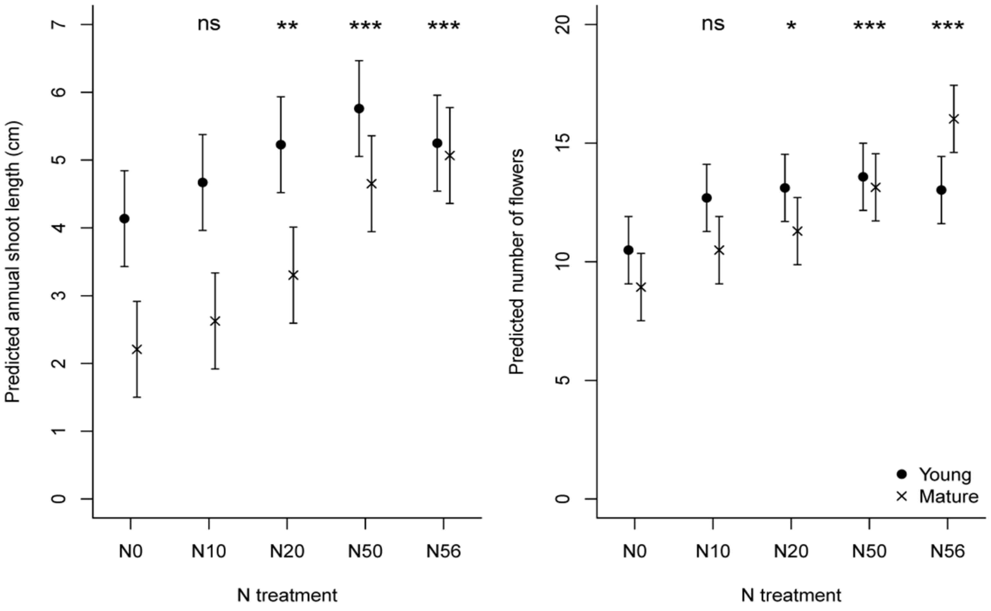

Figure 4.1. Predicted values (mean ± 95% confidence intervals) of annual shoot length (cm) and number of flowers of *Calluna* plants in the two differently aged heathland areas (young, mature) and the five N treatments (N0, N10, N20, N50, and N56). Significance levels of N treatments with respect to the control N treatment (N0) are indicated by *** ($0.001 > p$), ** ($0.01 > p > 0.001$), * ($0.05 > p > 0.01$), and ns ($p > 0.05$).

Table 4.1. LMM results for the effects of heathland age (young, mature) and N treatment (N0, N10, N20, N50, and N56) on *Calluna* annual shoot length (cm) and number of flowers. The interaction term (age x N treatment) was retained in the models only when significant. Standard deviations (SD) and variance components (%) of the random effects (identity of the heathland sites) are given. Df= degrees of freedom. Significant p-values are in bold face.

	Fixed effects				Random effects		
Response variable	Predictor variable	Df	F value	p value	Variable	SD	Variance
Annual shoot length	(Intercept)	1	343.62	**<0.001**	Heathland site	0.370	30.40
	Age	1	64.81	**<0.001**	Residual	0.847	69.60
	N treatment	4	20.43	**<0.001**			
	Age:N treatment	4	3.96	**0.006**			
Number of flowers	(Intercept)	1	1542.47	**<0.001**	Heathland site	0.398	16.53
	Age	1	2.03	0.158	Residual	2.010	83.47
	N treatment	4	14.78	**<0.001**			
	Age:N treatment	4	5.00	**0.001**			

4.3.2. Plant species composition

There were no significant changes in plant species composition in response to the increase in N load (Table 4.2). However, the *Calluna* life-cycle (age stand) showed a significant influence on plant species composition (Table 4.2; Fig 4.2). Young stands showed higher cover of graminoid species such as *Aira caryophyllea* L., *Carex nigra* (L.) Reichard, *Carex pilulifera* L., *Deschampsia flexuosa* (L.) Trin., *Festuca rubra* L., *Juncus squarrosus* L., and *Nardus stricta* L., as well as other perennial herbs including *Rumex acetosella* L., woody species such as *Cytisus purgans*, and non-vascular species like bryophytes (Table 4.3; Fig. 4.2). By contrast, mature stands were characterized by higher cover of woody species such as *Calluna vulgaris* L. (Hull), *Erica tetralix* L., and

Vaccinium myrtillus L., and lichens including *Cetraria islandica* (L.) Achar and *Cladonia* spp. P. Browne (Table 4.4; Fig. 4.2).

Table 4.2. Results of the permutational MANOVA testing for differences in plant species composition among the two heathland ages (young, mature) and the five N treatments (N0, N10, N20, N50, and N56). Df= degrees of freedom. Significant p-values are in bold face.

Factor	Df	Sum of squares	F value	p value
Age	1	0.48	8.67	**0.001**
N treatment	4	0.17	0.78	0.698
Age:N treatment	4	0.06	0.28	0.995
Residual	20	1.11		

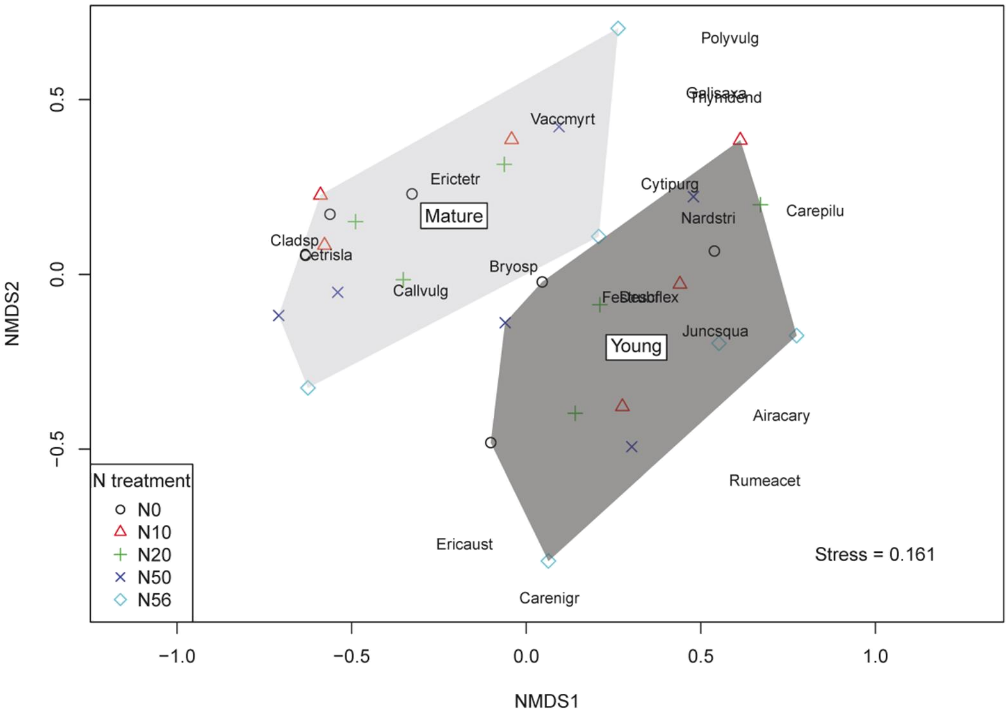

Figure 4.2. Plant species composition of the two heathland ages (young, mature) and the five N treatments (N0, N10, N20, N50, and N56) represented by the first two axes of the NMDS ordination. Permutational MANOVA revealed significant differences in species composition between the two heathland ages (see Table 4.2).

Table 4.3. Plant species cover (%) and total cover (%) in young heathlands for each N treatment (N0, N10, N20, N50 and N56). Data are observed means (SE) for the year 2014. AG (annual graminoid), PG (perennial graminoid), AF (annual forb), PF (perennial forb), W (woody), B (bryophyte), L (lichen).

		N treatment				
	Life-form	N0	N10	N20	N50	N56
Aira caryophyllea L.	AG	0.2 (0.1)	1.7 (0.8)	1.2 (0.7)	2.0 (1.5)	4.3 (1.7)
Agrostis capillaris L.	PG				0.2 (0.2)	
Briophyta Schimp.	B	11.8 (3.3)	17.1 (4.8)	15.3 (1.9)	13.3 (2.4)	4.3 (1.7)
Calluna vulgaris (L.) Hull	W	63.8 (3.9)	63.9 (4.0)	67.5 (3.8)	61.9 (5.2)	61.3 (3.9)
Carex asturica Boissier	PG	0.5 (0.4)	2.1 (1.5)	1.2 (0.7)	0.4 (0.4)	
Carex nigra (L.) Reichard	PG	1.9 (1.7)	0.5 (0.4)	1.1 (1.0)	1.9 (1.2)	2.6 (1.7)
Carex pilulifera L.	PG	1.8 (1.0)	1.6 (0.8)	1.8 (1.0)	2.3 (1.5)	9.4 (4.4)
Cetraria islandica (L.) Achar.	L	7.8 (4.0)	2.4 (0.8)	4.5 (2.4)	7.8 (4.1)	0.1 (0.0)
Cladonia spp. P. Browne	L	4.8 (4.0)	0.2 (0.1)	1.1 (0.5)	4.4 (1.7)	<0.1 (0.0)
Conopodium majus (Gouan) Loret	PF		0.1 (0.1)	0.3 (0.2)		
Cytisus purgans (L.) Spach	W	0.4 (0.4)	1.3 (1.1)	1.3 (1.3)	0.1 (0.1)	
Daboecia cantabrica (Huds.) Koch	W	0.1 (0.1)		0.1 (0.1)	0.2 (0.1)	
Deschampsia flexuosa (L.) Trin.	PG	2.0 (0.6)	2.6 (0.8)	2.8 (0.5)	2.3 (0.7)	1.5 (0.6)
Erica australis L.	W	1.8 (0.9)	0.9 (0.4)	0.4 (0.3)	5.2 (3.3)	2.6 (1.6)
Erica tetralix L.	W	6.4 (3.7)	1.9 (1.1)	3.1 (1.4)	11.6 (3.4)	11.3 (4.8)
Festuca rubra L.	PG	7.4 (1.4)	7.1 (2.0)	7.3 (2.9)	2.5 (1.1)	5.0 (1.9)
Galium saxatile L.	PF		0.1 (0.1)	0.1 (0.1)	<0.1 (0.0)	0.1 (0.1)
Hieracium pillosella L.	PF		0.2 (0.2)			
Jasione montana L.	AF					0.1 (0.1)
Juncus squarrosus L.	PG	2.6 (1.5)	2.8 (1.2)	4.2 (2.3)	11.4 (3.1)	6.2 (2.3)
Luzula spp. DC.	PG	0.1 (0.1)				
Nardus stricta L.	PG	6.5 (2.7)	3.3 (1.0)	9.1 (3.4)	7.2 (2.8)	3.9 (1.8)
Polygala vulgaris L.	PF	0.1 (0.1)	0.1 (0.1)	0.1 (0.0)		
Potentilla erecta (L.) Raeusch.	PF	0.1 (0.1)			1.3 (0.7)	0.5 (0.3)
Pterospartum tridentatum L.	W	0.1 (0.1)				
Rumex acetosella L.	PF	0.6 (0.4)	0.4 (0.3)	0.2 (0.1)	<0.1 (0.0)	0.6 (0.3)
Scilla spp. L.	PG					<0.1 (0.0)
Sedum album L.	PF		0.1 (0.1)	0.1 (0.1)		
Thymelaea dendrobium (Lam.) Endl.	PF	0.3 (0.3)	1.9 (0.8)	0.9 (0.7)		
Vaccinium myrtillus L.	W	9.0 (04.1)	6.6 (4.5)	11.1 (6.4)	7.9 (4.0)	1.5 (0.8)
Total cover		129.8 (22.2)	119.0 (9.0)	134.5 (15.2)	143.8 (14.0)	115.3 (13.5)

Table 4.4. Plant species cover (%) and total cover (%) in mature heathlands for each N treatment (N0, N10, N20, N50 and N56). Data are observed means (SE) for the year 2014. AG (annual graminoid), PG (perennial graminoid), AF (annual forb), PF (perennial forb), W (woody), B (bryophyte), L (lichen).

		N treatment				
	Life-form	N0	N10	N20	N50	N56
Aira caryophyllea L.	AG	<0.1 (0.0)	<0.1 (0.0)			
Agrostis capillaris L.	PG					0.3 (0.2)
Briophyta Schimp.	B	4.6 (1.8)	5.3 (1.6)	6.2 (1.8)	8.3 (3.4)	0.5 (0.2)
Calluna vulgaris (L.) Hull	W	81.9 (2.1)	78.3 (2.2)	78.1 (2.1)	79.8 (1.8)	81.7 (2.1)
Carex nigra (L.) Reichard	PG				0.4 (0.4)	<0.1 (0.1)
Carex pilulifera L.	PG	<0.1 (0.0)		<0.1 (0.0)		2.6 (0.9)
Cetraria islandica (L.) Achar.	L	11.0 (1.6)	10.4 (1.7)	13.3 (2.2)	13.8 (3.6)	2.0 (0.9)
Cladonia spp. P. Browne	L	7.5 (2.0)	9.3 (1.6)	11.6 (1.6)	11.5 (2.2)	0.2 (0.1)
Conopodium majus Gouan	PF	0.1 (0.1)	0.1 (0.0)			
Cytisus purgans (L.) Spach	W	0.1 (0.1)		0.1 (0.1)	<0.1 (0.0)	
Daboecia cantabrica (Huds.) Koch	W					0.6 (0.6)
Deschampsia flexuosa (L.) Trin.	PG	<0.1 (0.0)	0.1 (0.1)	0.3 (0.1)	0.3 (0.2)	2.6 (0.7)
Erica australis L.	W		0.3 (0.3)	0.3 (0.3)	0.8 (0.6)	
Erica tetralix L.	W	10.7 (5.2)	16.8 (8.3)	7.0 (3.6)	13.8 (7.7)	10.6 (4.4)
Festuca rubra L.	PG	0.4 (0.2)	1.1 (0.9)	1.4 (0.3)	1.4 (0.6)	5.6 (3.2)
Galium saxatile L.	PF		0.1 (0.1)	0.1 (0.1)	0.5 (0.5)	
Jasione montana L.	AF			0.1 (0.1)	0.2 (0.1)	0.7 (0.6)
Juncus squarrosus L.	PG	<0.1 (0.0)		0.6 (0.4)	0.1 (0.1)	0.2 (0.1)
Luzula spp. DC.	PG			0.1 (0.1)		
Nardus stricta L.	PG	0.1 (0.1)	0.2 (0.1)	0.1 (0.1)	0.9 (0.7)	0.4 (0.4)
Polygala vulgaris L.	PF		<0.1 (0.0)	<0.1 (0.0)	0.1 (0.1)	
Potentilla erecta (L.) Raeusch.	PF					1.1 (1.1)
Rumex acetosella L.	PF					<0.1 (0.0)
Thymelaea dendrobium (Lam.) Endl.	PF		0.4 (0.4)	0.6 (0.6)	0.6 (0.5)	
Vaccinium myrtillus L.	W	10.3 (5.1)	12.5 (5.9)	9.6 (4.5)	9.4 (4.8)	14.8 (5.1)
Total cover		126.9 (8.1)	134.5 (12.1)	129.3 (8.3)	142.0 (17.4)	123.8 (18.6)

4.3.3. *Total species richness*

No significant changes were found for total plant species richness in response to increased N loads (Fig. 4.3; Table 4.5). However, total plant species richness was significantly affected by *Calluna* life-cycle stage, with higher richness in young heathland stands (Fig. 4.3; Table 4.5).

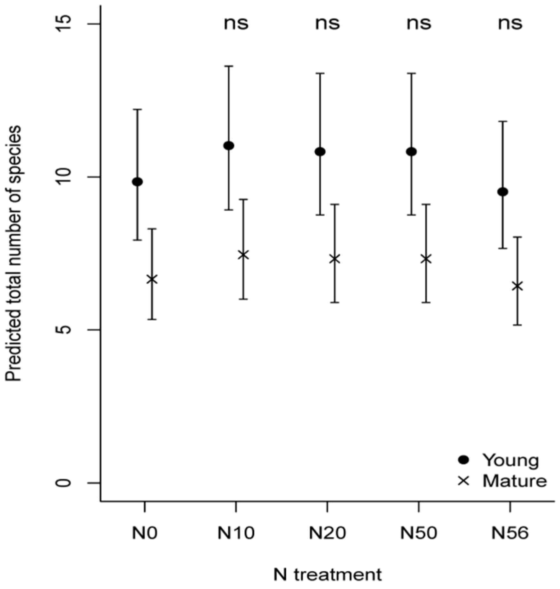

Figure 4.3. Predicted values (mean ± 95% confidence intervals) of plant species richness in the two differently aged heathland areas and the five N treatments. Significance levels of N treatments with respect to the control N treatment (N0) are indicated by *** ($0.001 > p$), ** ($0.01 > p > 0.001$), * ($0.05 > p > 0.01$), and ns ($p > 0.05$).

4.3.4. *Vascular life forms cover*

A significant increase in perennial forbs cover was found in response to enhanced N loads (Fig. 4.4; Table 4.5), only being significant in the low (N10) and chronic high N (N56) treatments (Fig. 4.4). Young stands showed significantly higher perennial forbs cover compared to mature ones (Fig. 4.4; Table 4.5).

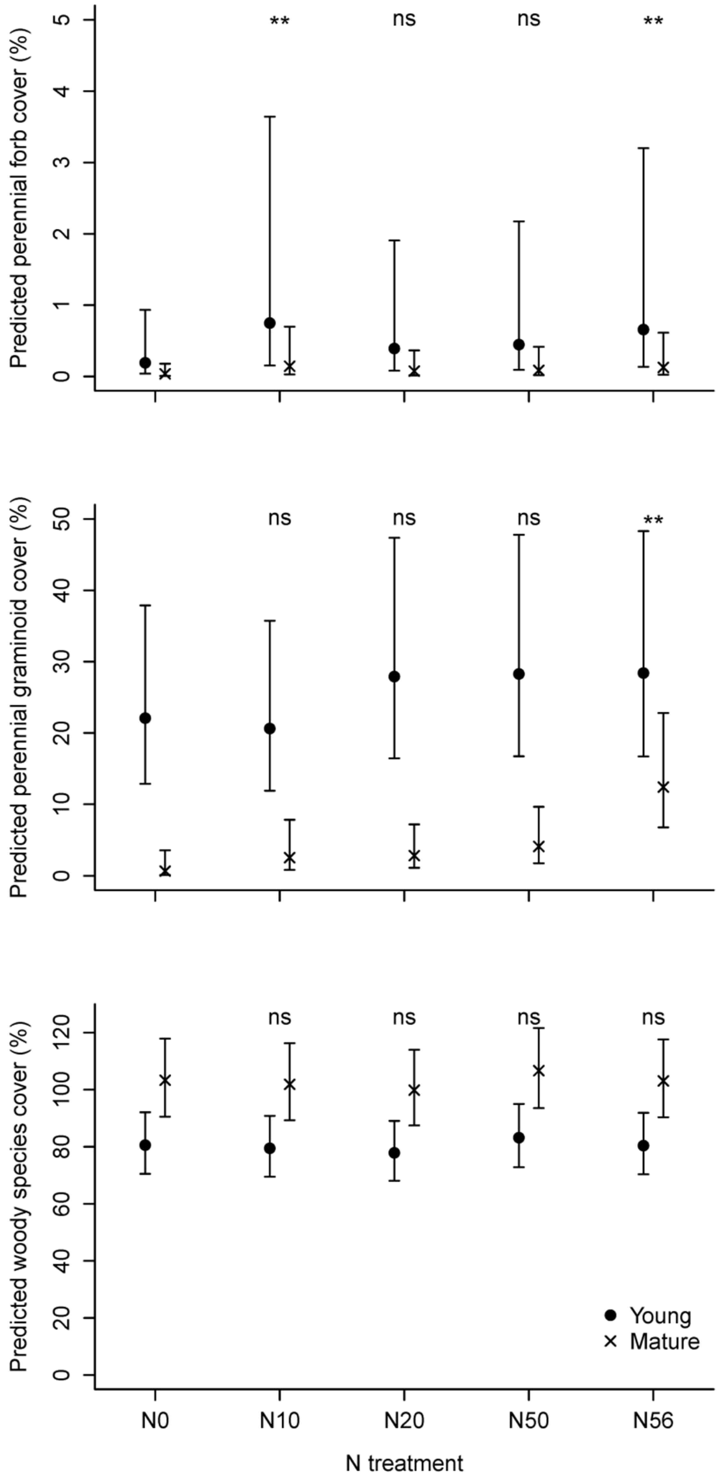

Figure 4.4. Predicted values (mean ± 95% confidence intervals) of the percentage cover (%) of perennial forbs, perennial graminoids, and woody species, in the two differently aged heathland areas and the five N treatments. Significance levels of N treatments with respect to the control N treatment (N0) are indicated by *** (0.001 > p), ** (0.01 > p > 0.001), * (0.05 > p > 0.01), and ns (p > 0.05).

Table 4.5. GLMM results for the effects of heathland age (young, mature) and N treatment (N0, N10, N20, N50, and N56) on the total number of plant species, and the percentage cover (%) of the plant life-forms: perennial forbs, annual forbs, perennial graminoids, annual graminoids, woody species, bryophytes, and lichens. The interaction term (age x N treatment) was retained in the models only when significant. Standard deviations (SD) and variance components (%) of the random effects (identity of the sampling plots nested within each heathland site) are given. Df= degrees of freedom. Significant p-values are in bold face.

	Fixed effects				Random effects		
Response variable	Predictor variable	Df	F value	p value	Variable	SD	Variance
Species richness							
Total no. of species	(Intercept)	1	553.11	**<0.001**	Heathland site	0.148	16.53
	Age	1	48.42	**<0.001**	Residual	0.750	83.47
	N treatment	4	1.12	0.352			
Vascular spp. cover							
Perennial forbs	(Intercept)	1	4.79	**0.031**	Heathland site	1.235	48.20
	Age	1	32.54	**<0.001**	Sampling plot	1.327	51.80
	N treatment	4	2.74	**0.034**	Residual	1.7×10^{-5}	6.8×10^{-4}
Annual forbs	(Intercept)	1	1447.77	**<0.001**	Heathland site	1.0×10^{-20}	0.00
	Age	1	11.77	**0.001**	Sampling plot	24.699	100.00
	N treatment	4	6.55	**<0.001**	Residual	3.2×10^{-22}	0.00
	Age:N treatment	4	3.21	**0.017**			
Perennial graminoids	(Intercept)	1	153.24	**<0.001**	Heathland site	0.384	15.43
	Age	1	60.40	**<0.001**	Sampling plot	0.198	7.95
	N treatment	4	2.70	**0.037**	Residual	1.909	76.62
	Age:N treatment	4	4.21	**0.004**			
Annual graminoids	(Intercept)	1	56895.73	**<0.001**	Heathland site	1.1×10^{-36}	0.00
	Age	1	53522.00	**<0.001**	Sampling plot	1.387	100.00
	N treatment	4	7912.08	**<0.001**	Residual	9.1×10^{-36}	0.00
	Age:N treatment	4	8180,93	**<0.001**			
Woody species	(Intercept)	1	5847.54	**<0.001**	Heathland site	0.096	6.53
	Age	1	62.82	**<0.001**	Residual	1.368	93.47
	N treatment	4	0.48	0.750			
Non-vascular spp. cover							
Bryophytes	(Intercept)	1	105.80	**<0.001**	Heathland site	0.344	13.54
	Age	1	25.58	**<0.001**	Residual	2.197	86.46
	N treatment	4	4.68	**0.002**			
Lichens	(Intercept)	1	103.69	**<0.001**	Heathland site	0.402	16.65
	Age	1	28.75	**<0.001**	Sampling plot	0.533	22.08
	N treatment	4	8.16	**<0.001**	Residual	1.479	61.27

The percentage cover of perennial graminoids showed a significant increase with enhanced N loads, but it was only significant under chronic high N treatment (N56) (Fig. 4.4; Table 4.5). Besides, the N-response of perennial graminoids was influenced by the heathland age, displaying significantly higher cover in young stands (Table 4.5). However, there was a different behaviour pattern in response to N loads between both heathland ages (significant 'age x N treatment' interaction; Table 4.5), since the cover of perennial graminoids progressively increased with increasing N loads in mature stands, whereas this cover increase was halted beyond N20 in young stands (Fig. 4.4).

Annual graminoids and forbs represented very low percentage of cover (less than 1.5% and 0.1% respectively). However, they showed significant cover increases in response to enhanced N loads (Table 4.5). Young heathlands showed higher annual herbs cover compared to mature ones, mainly related to the cover increase of *Aira cariophyllea* (Table 4.3).

The increase in N load did not show significant changes in woody species cover (Fig. 4.4; Table 4.5). However, significantly higher cover was found in mature stands compared to young ones (Fig. 4.4; Table 4.5).

4.3.5. Non-vascular life-forms cover

There was a significant negative effect caused by enhanced N loads on the cover of bryophytes, with a sharp decline in the chronic high N treatment (N56), regardless of the heathland life-cycle stage (Fig. 4.5; Table 4.5). Significantly higher cover of bryophytes was observed in young stands compared to mature ones (Fig. 4.5; Table 4.5).

The cover of lichens was negatively affected under the chronic high N treatment (N56) in both young and mature stands (Fig 4.5; Table 4.5), although mature heathlands showed significantly higher lichen cover compared to young ones (Fig. 4.5; Table 4.5).

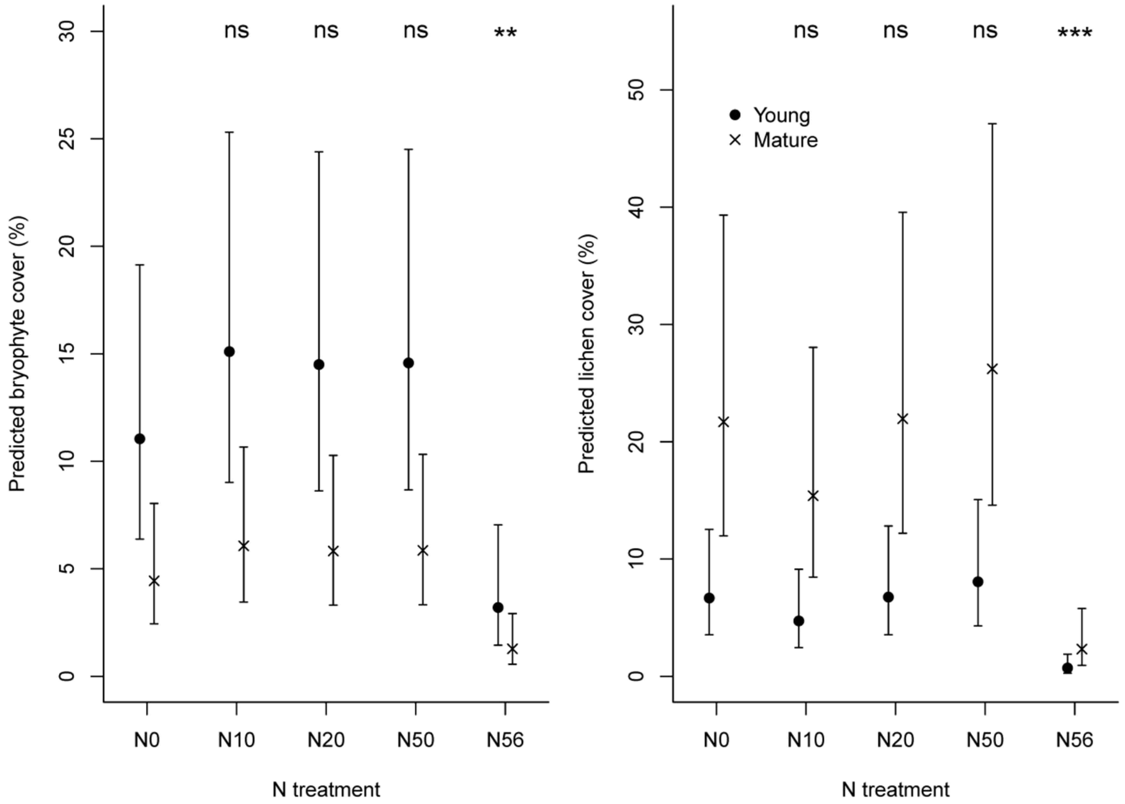

Figure 4.5. Predicted values (mean ± 95% confidence intervals) of the percentage cover (%) of bryophytes and lichens in the two differently aged heathland areas and the five N treatments. Significance levels of N treatments with respect to the control N treatment (N0) are indicated by *** (0.001 > p), ** (0.01 > p > 0.001), * (0.05 > p > 0.01), and ns (p > 0.05).

4.4. Discussion

4.4.1. *Effects of increased N loads*

The increased N depositions in heathlands are able to alter *Calluna vulgaris* vital rates (Britton and Fisher, 2008; Cuesta et al., 2008; Southon et al., 2013). As we hypothesized, there was a dose-related increase in *Calluna* vital rates such as current year's shoot length and flowering in response to short-term N loads. Similar increases were also observed in other N fertilization studies carried out in heathlands in NW Spain (Cuesta et al., 2008) and in central-northern European heathlands (Lee and Caporn, 1998; Britton and Fisher, 2008; von Oheimb et al., 2010; Southon et al., 2012; Bähring et al., 2017). This positive effect on *Calluna* shoot growth and flowering would demonstrate that the productivity of *Calluna* is primarily N-limited (Lee and Caporn, 1998; Carroll et al., 1999; von Oheimb et al., 2010), and higher N availability implies changes in its vital rates (Cuesta et al., 2008; Southon et al., 2012, 2013). The response of *Calluna* shoot growth to increased N loads was age-related, displaying longer shoots in young stands due to the higher rates of plant productivity in the building phase (Jones and Power, 2015). However, there was an opposite effect on *Calluna* shoot growth and flowering under the chronic high N treatment (N56) in young stands. Besides, young heathlands also showed a significantly greater *Calluna* shoot N content under these chronic high N loads [Chapter 3]. The coincidence of higher shoot N content together with lower shoot growth under chronic N inputs would indicate the existence of N-toxicity status in plants (Timmer, 1991; Sheppard et al., 2008), and would confirm our original hypothesis about the greater negative effects of chronic N inputs.

The effects associated to higher N availability were also observed at plant community level, mainly in terms of changes in the structure and species composition, as were observed in a wide range of studies carried out in central and northern European heathlands (Van den Berg et al., 2005; De Graaf et al., 2009; Southon et al., 2012, 2013; Fagúndez, 2013; Field et al., 2014; Stevens et al., 2016). However, in the Cantabrian Mountains heathlands, neither plant community composition nor plant species richness showed significant changes in response to increased N loads. This could be explained because the heathlands located at the southern-most distribution area have been frequently affected by stress drivers as drought events during summer months (Meyer-Grünefeldt et al., 2016) and historically perturbed by recurrent burning (Marcos et al., 2009). Therefore, the dominant woody species have developed resistance strategies to successfully respond to these perturbations (Calvo et al., 2002a, 2007; Camac et al., 2013), as well as resilience mechanisms to recover the pre-disturbed structure and composition by autosuccession processes (direct regeneration) (Calvo et al., 2002b). In this study, the differences in plant species richness and community composition were only shown between heathland ages, with higher plant species richness in young stands, as was also observed by Calvo et al. (2007). The opened canopy patches due to the low dwarf-shrubs cover in young stands facilitated the colonization of N-tolerant species such as graminoids and forbs (Bobbink et al., 2010; Friedrich et al., 2011b; Camac et al., 2013), and contributed to maintaining higher plant species richness compared to mature stands. Thus, it seems that the building phase after management is the most likely point in the heathland life-cycle stage for changes in species composition (Britton et al., 2000; Friedrich et al., 2011b).

Life forms cover was another community structural parameter affected by increased N loads. The percentage cover of annual graminoids, annual forbs and perennial forbs was increased in response to higher N loads, although they represented less than 1% cover. Particularly, the increase in annual species cover could be related to the favourable non-N-limited conditions for seedling recruitment associated to higher soil N availability in N-poor heathland soils (Power et al., 2001; Barker et al., 2004). By contrast, perennial graminoids represented a noteworthy life form group in terms of abundance, which was significantly increased under chronic high N load (N56). Graminoid species have been reported as nitrophilous plants that are expected to expand with higher N availability (Calvo et al., 2005; Bobbink et al., 2010; Friedrich et al., 2011b). In this study, the species responsible for the spread of perennial graminoids were mainly *Carex pilulifera* in young stands and *Carex pilulifera*, *Deschampsia flexuosa*, and *Festuca rubra* in mature stands, as was also shown in other studies (Calvo et al., 2002a; Southon et al., 2013; Britton et al., 2017). Regardless of N load, the cover of perennial graminoids was higher in young stands due to the rapid colonization of the opened gaps in the woody species canopy (Barker et al., 2004; Friedrich et al., 2011b; Camac et al., 2013). We did not observe changes in woody species cover in response to increased N loads, which could be explained by higher resistance and resilience of southern-most *Calluna* populations in response to environmental perturbations (Calvo et al., 2005, 2007) compared to central and northern European heathlands.

The main negative effect of increased N loads on heathland structure was the cover reduction of N-sensitive non-vascular vegetation (bryophytes and lichens), which could be related to tissue N toxicity caused by high N availability (Carroll et al., 1999; Pearce and Van der Wal, 2002). Bryophyte layer has been reported to be the main N sink for atmospheric N depositions in heathlands (Friedrich et al., 2011a), which could behave as the earliest ecosystem compartment affected by an increase in airborne N inputs (Choudhary et al., 2016). However, we observed that the reduction in bryophyte cover only occurred under chronic N loads (N56). This could be explained by slow N cycling in montane heathlands due to the short growing season and low temperatures, displaying delayed N-related effects compared to lowland heaths (Pearce and Van der Wal, 2002; Britton et al., 2008). Lichen species have also demonstrated to be highly sensitive to enhanced N availability (Remke et al., 2009; Phoenix et al., 2012; Field et al., 2014), but we only observed a strong decline close to the virtual disappearance in response to chronic N inputs (N56), likely due to tissue N saturation caused by the direct absorption of airborne N inputs (Nielsen et al., 2014). The decline in cryptogams was of essential importance for heathland nutrient cycling because bryophytes and lichens contribute to immobilizing atmospheric N depositions through surface absorption (Nielsen et al., 2014; Choudhary et al., 2016), and thus neutralize the harmful effects associated to these N inputs (Pilkington et al., 2007). The decline in bryophytes and lichens was described by Bobbink et al. (2010) as one symptom of the exceedance of N critical load for dry heathlands (10-20 kg N ha^{-1} yr^{-1}), although we only observed a cryptogam decline under cumulative N loads (N56), similar to Phoenix et al. (2012).

4.4.2. Implications for heathland management

Our findings showed that higher N inputs had no effect on plant species richness, which would demonstrate that our montane heathlands could be highly resistant to environmental perturbations (Calvo et al., 2007). However, we observed that the rejuvenated heathland achieved by the application of prescribed burning resulted in higher plant species richness due to the recovery of pre-managed plant species and the addition of new fast-growing species. This fact would demonstrate that prescribed burning may be an appropriate management tool to preserve the biodiversity of Cantabrian heathlands (Calvo et al., 2012; Fagúndez, 2013). However, a high recurrence (< 10 years) of prescribed burning could potentially imply negative effects on lichens due to the lack of protection for direct N inputs by the dwarf-shrub canopy (Pilkington et al., 2007; Britton et al., 2008). For this reason, a long-term cycle (20-30 years; Calvo et al., 2002a, 2012) of prescribed burning should be applied in these montane heathlands to prevent of the degenerate phase from occurring (Gimingham, 1972); and remove the excess of N inputs that have been accumulated in the vegetation and litter pools (Barker et al., 2004; Marcos et al., 2009; Jones et al., 2017). Other studies carried out in the Cantabrian Mountains have reported that cutting management is not an appropriate tool to preserve montane heathlands due to induced changes in plant community composition through the replacement of the dominant species (Calvo et al., 2002a; 2012).

4.5. Conclusions

The present study examined the effects of increased N loads on plant community structure and composition of the montane heathland ecosystem, as well as on the vital rates of *Calluna vulgaris*. At the *Calluna* dominant-species level, we observed positive responses in *Calluna* shoot growth and flowering with short-term N loads, although decreased trends were observed with high chronic N loads (N56; 10 years), which could indicate the earliest signs of N saturation. At community level, there were no changes in plant species richness and composition in response to increased N loads, likely due to the resistance and resilience strategies of the heathland dominant species (*Calluna vulgaris* and *Erica tetralix*) to respond to recurrent perturbations. Enhanced N loads resulted in an increase in perennial and annual graminoids and forbs cover. The main negative effects were observed in non-vascular species, bryophytes and lichens, with strong cover declines under chronic high N load (56 kg N ha^{-1} yr^{-1}; 10 years), likely due to phytotoxicity related to N inputs. There were significant differences in vegetation structure and composition in relation to heathland age. Young heathlands showed different plant community composition and higher species richness compared to mature ones, since the opened gaps in the dwarf-shrub canopy in young stands allowed the colonization of graminoid and forb species. We proposed prescribed burning as an appropriate management tool to preserve the biodiversity of Cantabrian Mountains heathlands, as it contributes to maintaining these communities in a life-cycle stage previous to the degenerate phase.

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CHAPTER 5

Nitrogen critical loads for montane heathlands at their southern-most distribution limit in Europe (NW Spain)

Abstract

The nitrogen (N) critical load is a valuable tool to assess the harmful effects of N depositions on ecosystem health. The present study established the N critical load for montane *Calluna vulgaris* heathlands located at their southern-most distribution limit in two *Calluna* life-cycle stages: young- and mature-phase. We experimentally simulated four levels of N fertilization: 0, 10, 20, and 50 kg N ha^{-1} yr^{-1} over background deposition (4.6 kg N ha^{-1} yr^{-1}) for 3 years in each heathland age. We performed critical load estimations based on significant N-related changes in heathland vegetation composition and structure, as well as in heathland functioning by the assessment of several ecosystem response variables. Young heathlands showed an increase in *Calluna vulgaris* flowering and shoot N content at 14.6 kg N ha^{-1} yr^{-1}; whereas mature heathlands showed a significant increase in *Calluna vulgaris* flowering at 14.6 kg N ha^{-1} yr^{-1}. These critical thresholds demonstrated that (1) the heathland age was not a determining factor in the estimation of N critical loads in montane heathlands, and (2) the N critical load was 14.6 kg N ha^{-1} yr^{-1} for both young and mature stands in montane heathlands.

5.1. Introduction

During the last century, intensive agriculture and fossil fuel combustion have changed global emissions and depositions of reactive nitrogen (Nr) (Galloway et al., 2004). Elevated N inputs into terrestrial ecosystems have been related to harmful effects on the health of ecosystems (Bleeker et al., 2011; Galloway et al., 2013). The magnitude of these effects mainly depends on the specific N-sensitivity of each ecosystem-type (Fenn et al., 2010). Particularly, ecosystems adapted to low levels of N availability (low N-status) such as montane heathland systems are more vulnerable to increased atmospheric N depositions (Britton et al., 2008; Bobbink and Hettelingh, 2011).

One valuable tool to determine ecosystem sensitivity to changes in N availability is the critical load concept (Rodríguez-Lado and Macías, 2006; Pardo et al., 2011), which was set by the Gothenburg Protocol due to the importance of defining critical thresholds for acidification and eutrophication effects of N depositions in order to protect ecosystems and their services (Roth et al., 2017). It was defined as "a quantitative estimate of deposition of one or more pollutants below which significant harmful effects on specified elements of the environment do not occur according to present knowledge" (Nilsson and Grennfelt, 1988). Critical loads can be determined: (1) based on empirical evidences through experimentally induced or observed changes in ecosystems at different levels of N inputs, e.g. plant species composition (Belyazid et al., 2011; Pardo et al., 2011; Bowman et al., 2012; Henry and Aherne, 2014; Roth et al., 2017), or (2) based on mass balance models or equations which balance the long-term chemical N inputs and outputs (Henry and Aherne, 2014; Hall et al., 2015). The critical thresholds are given as ranges of N depositions in order to reflect the variation of site-specific ecosystem response, determined by the influence of soil abiotic conditions and historic land use and management practices (Bobbink and Roelofs, 1995). Thus, exceedance of critical loads by current or future nitrogen loads indicates risks for adverse effects on ecosystem health (Pardo et al., 2011; Henry and Aherne, 2014).

Critical loads are assigned to EUNIS habitat classes to enable consistency of habitat terminology. Initially, the critical load of N for dry heathlands (F4.2 EUNIS class) was established in 15-20 kg N ha^{-1} yr^{-1} (Bobbink and Roelofs, 1995) based on observed changes in the structure and function of the heathland ecosystem reported by a wide range of publications. Afterwards, the critical loads for N deposition in dry heathlands were empirically established in the range 10-20 kg N ha^{-1} yr^{-1} (Bobbink and Hettelingh, 2011). However, a continuous revision of N critical loads for dry heathlands has been proposed, suggesting that lower critical thresholds must be reconsidered and updated en view of the results of recent studies about N deposition impact (Remke et al., 2009; Hall et al., 2015). This assumption is based on the recently observed harmful effects below the lower limit of current N critical load in dry heathlands (10 kg N ha^{-1} yr^{-1}), such as a decline in plant species richness (8.8 kg N ha^{-1} yr^{-1}; Tipping et al., 2013) or a reduction in lichen abundance (7.7 kg N ha^{-1} yr^{-1}; Phoenix et al., 2012). This fact would demonstrate the high sensitivity of heathland ecosystems to increased atmospheric N depositions (De Graaf et al., 2009). In particular, the dry heathlands of montane areas have demonstrated to be more sensitive to atmospheric N depositions compared to lowland heaths (Britton et al., 2008; Britton and Fisher, 2008). Furthermore, *Calluna* heathlands located at their southern-most distribution limit (Cantabrian Mountains, NW Spain) are expected to respond differently to global change perturbations such as enhanced airborne N depositions (Meyer-Grünefeldt et al., 2016), since they are well adapted to frequently respond to other environmental stresses such as water shortage during drought events and extreme low temperatures in winter.

The heathland life-cycle stage could also be considered as one essential factor in the estimation of N critical load. It has been reported that young heathlands are more susceptible to manifesting deleterious effects related to environmental changes such as enhanced N depositions (Britton and Fisher, 2008; Jones and Power, 2015). Thus, it is possible that young stands may be more vulnerable to enhanced N availability at lower N loads compared to mature ones. The higher N-sensitivity of young stands is mainly attributed to higher *Calluna* productivity and shoots N demand to support the faster growth rates in this life-cycle stage (Jones and Power, 2015), as well as to changes in the heathland vegetation structure through the replacement of the dominant shrubby species by N-tolerant and fast-growing grasses (Britton et al., 2001).

The aim of this study was to determine the N critical load for montane heathlands in the Cantabrian Mountains based on changes in vegetation structure and composition, and the ecosystem functions. We proposed that the N critical threshold be established at two *Calluna vulgaris* heathland life-cycle stages: young- and mature-phase; since the stand age might influence its susceptibility to N load (Reynolds et al., 1998; Britton et al., 2001; Jones and Power, 2015). We hypothesized that montane heathlands could be more vulnerable to increased N depositions compared to lowland heaths (Britton et al., 2008) We also hypothesized that young heathlands could exhibit lower N critical loads, since they would be more sensitive to N depositions due to the stronger effects in response to higher N availability (Jones and Power, 2015).

5.2. Materials and methods

5.2.1. Study area

We selected a study area composed by three homogeneous *Calluna*-heathland sites in the Cantabrian Mountains range (NW Spain): Riopinos I (1660 m a.s.l., 43°02'N, 5°24'W), Riopinos II (1560 m a.s.l., 43°02'N, 5°26'W), and San Isidro (1620 m a.s.l., 43°03'N, 5°21'W) (Fig. 5.1). The climatic, soil and vegetation characteristics of these study sites were previously defined in Chapter 3 and Chapter 4.

N background deposition in the study area has been calculated at 4.6 kg N ha^{-1} yr^{-1} in the San Isidro study site (Calvo-Fernández et al., 2017). This background deposition value has also been used in the calculation of N critical loads in the other two study sites (Riopinos I and Riopinos II) because they are less than 8 km apart, and therefore, differences in background depositions should be minimal.

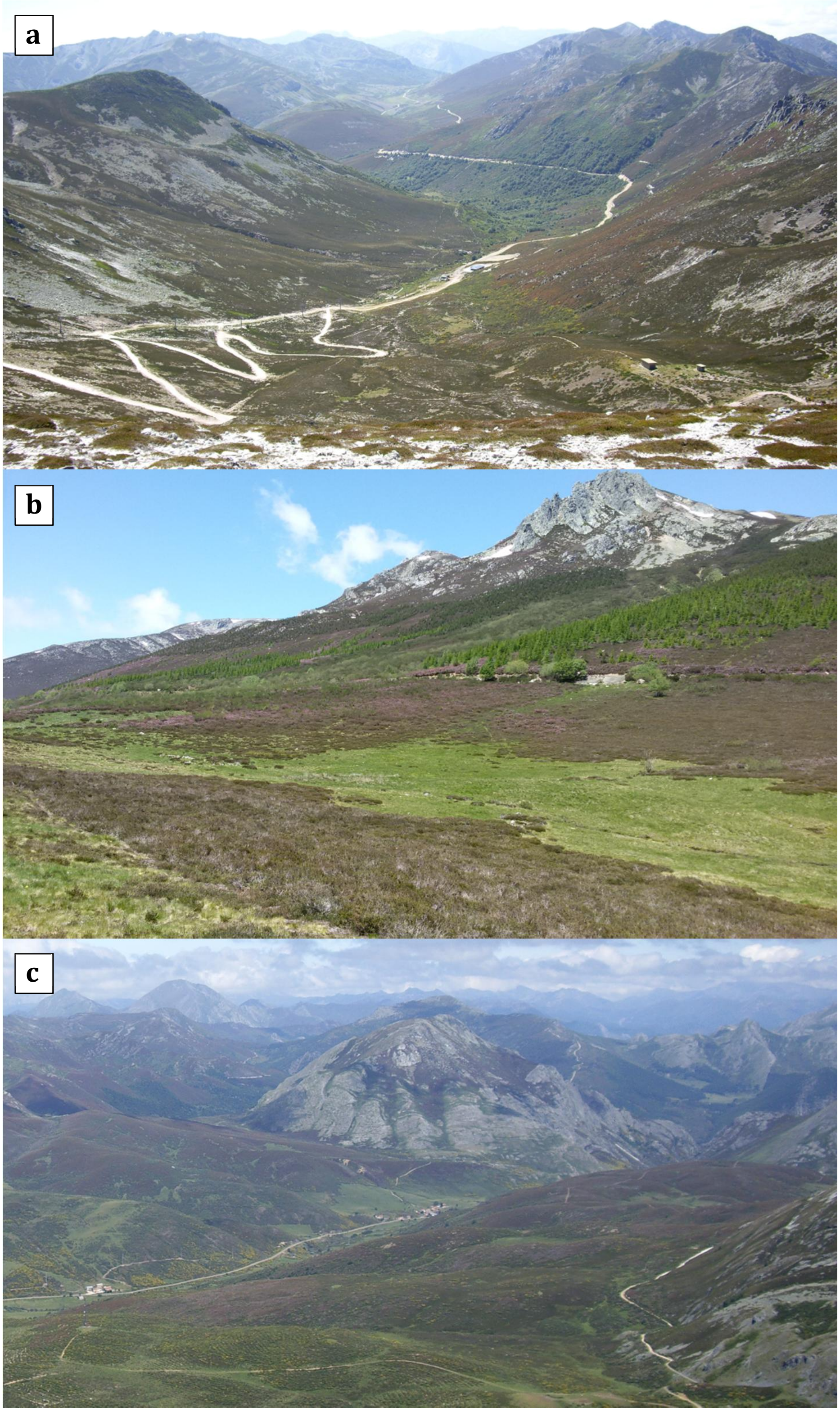

Figure 5.1. General location of (a) Riopinos I, (b) Riopinos II, and (c) San Isidro study sites.

5.2.2. Experimental design

We selected two heathland stands of different ages in each study site: (1) rejuvenated stands through prescribed fire in 2005 (i.e., young or building-phase; Gimingham, 1972), and (2) mature stands after 30-40 years of land use abandonment (i.e., mature-phase; Gimingham, 1972). We established a total of 72 2 m x 2 m plots in 2013 and performed a manipulative experiment consisting of four randomly-assigned N fertilization treatments over background deposition of 4.6 kg N ha^{-1} yr^{-1} (i.e., 3 replicated plots per N treatment, age class, and site): 0 kg N ha^{-1} yr^{-1} (N0 treatment), 10 kg N ha^{-1} yr^{-1} (N10 treatment), 20 kg N ha^{-1} yr^{-1} (N20 treatment), and 50 kg N ha^{-1} yr^{-1} (N50 treatment). Fertilization consisted of granules of ammonium nitrate (Fertiberia S.A.; 27% NH_4NO_3 purity) added monthly by hand from June to November.

5.2.3. Sampling and analytical methods

We annually measured a wide range of plant and soil response variables to increasing N loads for each plot in 2014 and 2015 (one and two years after N treatments), which are coincident with the soil and vegetation parameters measured in Chapter 3 and Chapter 4 to evaluate the effect of increased N loads. Vegetation response variables were: vegetation cover at two levels, single species (31 plant species) and life-forms (annual forbs, perennial forbs, annual graminoids, perennial graminoids, woody, bryophytes, and lichens); as well as plant species richness, *Calluna* flowering, current year's *Calluna* shoot length, *Calluna* shoot and litter N and P contents, and *Calluna* and litter N:P ratios. Soil response variables were: extractable NH_4^+ and NO_3^-, total N, organic C, available P, microbial biomass N and C, microbial C:N ratio, and enzymatic activities (acid phosphatase, urease, and β-glucosidase). The sampling and analytical procedures of these soil and vegetation response variables were previously detailed in Chapter 3 and Chapter 4.

5.2.4. Calculation of N critical loads

The empirical N critical loads were estimated for each heathland life-cycle stage separately (young and mature phase). To analyse the effects of increased N loads on the soil and vegetation response variables for each heathland age, we performed a two-way repeated measures ANOVA, with ‘year’ as repeated measure (2014 and 2015) and ‘N treatment’ as a factor. The differences between the treatment levels of N addition (N10, N20, and N50) with respect to background deposition (N0, control treatment) for those dependent variables with significant ‘N treatment’ effect were analysed using a post-hoc Tukey’s test. The N critical loads were determined by the lowest N treatment at which the response variable showed a significant change with respect to the control N treatment (N0). All statistical analyses were performed using SPSS 20.0 (SPSS Inc., Chicago, IL, USA).

5.3. Results and Discussion

For young heathlands, significant ‘N treatment’ effects were only found for *Calluna* vital rates such as number of flowers and current year’s *Calluna* shoot length, as well as functional traits such as *Calluna* shoot N and P contents, and litter N content (Table 5.1). By contrast, we did not observe significant N-related changes in other heathland functioning properties corresponding to soil response variables, since the effects on the structure and composition of the vegetation generally take precedence over detectable changes in soil parameters in response to increased N loads (Bowman et al., 2006). This fact points out that N critical loads can be better estimated using vegetation properties (Belyazid et al., 2011). However, it may also suggest that soil properties in montane heathlands could be highly resistant to harmful changes in response to enhanced N availability, and therefore, according to our results, more than 3 years of N inputs would be needed to establish the N critical threshold for soil properties.

Table 5.1. Direction of response (↑ increased or ↓ decreased) and significance level [*** ($p < 0.001$), ** ($0.01 > p > 0.001$), * ($0.05 > p > 0.01$), and ns ($p > 0.05$)] of different N treatments (N10, N20 and N50) with respect to the control N treatment (N0) for *Calluna* flowering (no. flowers per shoot), current year's shoot length of *Calluna* (cm), *Calluna* shoot N content (%), *Calluna* shoot P content (%), and litter N content (%) in young *Calluna* stands. Significance level of 'N treatment' effect for each response variable is presented as p-value.

	N treatment			
	N10	N20	N50	p-value
No. flowers	↑ **	↑ ***	↑ ***	0.000
Calluna shoot length	ns	↑ **	↑ ***	0.000
Calluna shoot N content	↑ ***	↑ ***	↑ ***	0.000
Calluna shoot P content	ns	↑ *	↑ *	0.017
Litter N content	ns	ns	↑ *	0.030

All the selected response variables in young heathlands showed an increase in response to higher N loads, but the significant effects with respect to background deposition (4.6 kg N ha^{-1} yr^{-1}; N0 treatment) were found at 14.6 kg N ha^{-1} yr^{-1} load (N10 treatment) for

Calluna flowering and shoot N content; at 24.6 kg N ha^{-1} yr^{-1} for *Calluna* shoot growth and shoot P content (N20 treatment); and at 54.6 kg N ha^{-1} yr^{-1} for litter N content (N50 treatment) (Table 5.1). Thus, according to our findings, the N critical load for young montane heathlands could be established at the lowest N deposition level at which any change was identified, i.e. 14.6 kg N ha^{-1} yr^{-1}. However, this N critical load is mainly based on damaging changes in *Calluna* biochemistry (increased shoot N content), since the alteration of *Calluna* phenology (higher shoot flowering) should not be considered as an important harmful effect. By contrast, the increase in *Calluna* flowering in response to higher N availability could be identified as a positive impact for its reproductive potential, since the increased pool size of *Calluna* seeds within the soil seed bank could facilite the sucessful recovery of the heathland-dominant *Calluna vulgaris* from frequent and intense perturbations through higher seedling recruitment.

Although the increase in *Calluna* shoot N content apparently also seems to be a positive impact related to higher N availability, it has been widely reported that changes in *Calluna* shoot biochemistry will have negative consequences for heathland health, being considered as one of the most sensitive indicators of the impact of N depositions (Phoenix et al., 2012). The observed increase in *Calluna* shoot N content at 14.6 kg N ha^{-1} yr^{-1} could have been the trigger for the subsequent changes in *Calluna* shoot length and *Calluna* shoot P content at 24.6 kg N ha^{-1} yr^{-1}, as well as in litter N content at 54.6 kg N ha^{-1} yr^{-1}. The growth of *Calluna* shoots is initially limited by N (Lee and Caporn, 1998; von Oheimb et al., 2010), and therefore, the increase in shoot N content could result in stronger stimulation of current year's shoot length (Carroll et al., 1999). Besides, higher shoot N content implies higher P demand due to plant nutrient stoichiometry (Rowe et al., 2008; Friedrich et al., 2011; Villalón, 2014; Jones and Power, 2015), enhancing competition for soil available P in nutrient poor soils typical of heathlands (von Oheimb et al., 2010). The N-enriched *Calluna* shoots will also be incorporated into the litter layer, which will result in an increase in litter N content (Pilkington et al., 2005; Jones and Power, 2012; Villalón, 2014), therefore contributing to disrupting the soil biogeochemical N by alterations in microbial mineralization processes (Lee and Caporn, 1998; Stevens et al., 2011). Besides, the increase in *Calluna* shoot N content has been related to enhanced sensitivity to stress factors such as drought, frost injury, and heather beetle attack (Taboada et al., 2016; van Voorn et al., 2016). Thus, the increase in *Calluna* shoot N content could mean a cascade of deleterious effects for heathland ecosystem health (Phoenix et al., 2012).

For mature heathlands, significant 'N treatment' differences were only found for *Calluna* flowering, current year's *Calluna* shoot length, *Calluna* shoot N content and litter N:P ratio (Table 5.2). All these variables showed an increase in response to higher N loads, but the significant differences with respect to background deposition (4.6 kg N ha^{-1} yr^{-1}; N0 treatment) were found at a deposition load of 14.6 kg N ha^{-1} yr^{-1} (N10 treatment) for *Calluna* flowering; and at 24.6 kg N ha^{-1} yr^{-1} for *Calluna* shoot growth, *Calluna* shoot N content and litter N:P ratio (N20 treatment) (Table 5.2). Thus, according to these findings, the N critical load for mature montane heathlands could be established in the range of 10-20 kg N ha^{-1} yr^{-1}, as was also estimated for young heathlands. Furthermore, our results also showed that montane heathlands are more sensitive to increased N loads than other mature lowland and upland heathlands of central and northern Europe, where they have shown significant increases in *Calluna* shoot N content at higher N loads than 30 kg N ha^{-1} yr^{-1} (Power et al., 1998; Barker et al., 2004; Pilkington et al., 2005; Britton and Fisher, 2008; von Oheimb et al., 2010; Southon et al., 2012).

Therefore, it suggests that montane heathlands at their southern-most distribution limit could be more susceptible to N-induced changes in *Calluna* shoot nutrient content. Furthermore, it has been reported by modelled estimations that current atmospheric N depositions in Cantabrian Mountains heathlands could be exceeding the critical threshold for this ecosystem type (García-Gómez et al., 2014).

Table 5.2. Direction of response (↑ increased or ↓ decreased) and significance level [*** ($p < 0.001$), ** ($0.01 > p > 0.001$), * ($0.05 > p > 0.01$), and ns ($p > 0.05$)] of different N treatments (N10, N20 and N50) with respect to the control N treatment (N0) for *Calluna* flowering (no. flowers), current year's shoot length of *Calluna* (cm), *Calluna* shoot N content (%), and litter N:P ratio in mature *Calluna* stands. Significance level of 'N treatment' effect for each response variable is presented as p-value.

	N treatment			
	N10	N20	N50	p-value
No. flowers	↑ **	↑ ***	↑ ***	0.000
Calluna shoot length	ns	↑ ***	↑ ***	0.000
Calluna shoot N content	ns	↑ *	↑ ***	0.000
Litter N:P ratio	ns	↑ *	↑ *	0.013

Our results are in accordance with the current N critical load for dry European heathlands, which has been established in the range from 10 to 20 kg N ha^{-1} yr^{-1} (Bobbink and Hettelingh, 2011), and confirmed by Hall et al. (2015) for UK heathlands. However, there were differences related to the ecosystem indicators used for the estimation of these N critical thresholds. In our study, we identified the plant biochemistry and the *Calluna* vital rates as the more accurate indicators for the establishment of N critical load in heathlands. This fact confirms the idea of Posch et al. (2011) that critical load is considered a site-specific ecosystem property.

5.4. Conclusions

In this study we estimated the N critical loads for montane heathlands at their southernmost distribution limit depending on heathland life-cycle stage (i.e., young- and mature-phase). For young heathlands, the first symptoms of harmful effects related to N depositions were modifications in *Calluna* vital rates through an increase in the number of flowers per shoot, and in *Calluna* biochemistry through an increase in shoot N content, both above 14.6 kg N ha^{-1} yr^{-1}; whereas for mature heathlands was an increase in *Calluna* flowering above 14.6 kg N ha^{-1} yr^{-1}. Therefore, there were no differences related to *Calluna*-heathland age in the estimated N critical load for young and mature stands. These estimations displayed that the current N critical loads in montane heathlands are established within the range 10-20 kg N ha^{-1} yr^{-1}.

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Discussion

The atmospheric N depositions in the Cantabrian Mountains are influenced by the seasonal dynamic of the emission sources and volume of precipitation (Cape et al., 2015; Izquieta and Rojano, 2016), as well as chemical and physical N scavenging processes from the atmosphere (de Souza et al., 2015). The highest bulk N deposition (4.6 kg N ha^{-1} yr^{-1}) was observed in the areas with the highest annual precipitation (1645 mm). In the San Isidro study site, there was a prevalence of N oxidized forms (NOx) in airborne depositions, reflecting the great influence of airborne NO_X emissions from industrialized areas in the northern region of Asturias, mainly from coal mines, coal power plants, and other factories with combustion processes (Cuevas et al., 2014). However, the other study site, La Majúa, showed the typical pattern of an intermediate situation between industrialized areas and agricultural areas surrounded by croplands and pasturelands with high NH_3 volatilization from nitrogenous fertilizers and livestock excreta (Asman et al., 1998; Zhan et al., 2015). The prevalence of NO_X depositions could reduce the threat of harmful effects on ecosystem health, since prevailing NH_4^+ depositions would have a greater impact on heathland vegetation through adverse phytotoxic effects associated to soil acidification (De Graaf et al., 2009; Stevens et al., 2011).

Understanding the allocation patterns of atmospheric N depositions and storage within the different ecosystem compartments could be of great importance to evaluate the potential impacts of current N inputs (Friedrich et al., 2011a). The use of ^{15}N tracer was a useful tool to know the distribution and fate of atmospheric N depositions within the ecosystem compartments, because it provides information about the N cycle dynamic, input/output flows and ecosystem storage capacity (Carreira et al., 2010). It was found that these heathlands stored 72% of atmospheric N depositions during a time period less than 1 year, and acted as a source for a time period between 1-2 years because they only retained 5% of N inputs. The great output of N during the second year could be due to denitrification fluxes created by anoxic conditions in waterlogged soils during the snow cover period (Wolf and Russow, 2000). Friedrich et al. (2011a) also demonstrated that heathlands in central Europe (NW Germany) acted as N source in the second year. During the first year the organic horizon in Cantabrian heathlands acted as the major N sink (~ 47%). This finding was in agreement with the results of other studies on N allocation patterns carried out in UK heathlands, where the organic horizon acted as the main ecosystem sink for N inputs (Power et al., 1998; Carroll et al., 1999; Pilkington et al., 2005b), but different from other central and northern European heathlands where the moss layer acted as the main sink for N inputs (Curtis et al., 2005; Friedrich et al., 2011a). Regarding *Calluna* aboveground biomass, it represented a small sink for N inputs as was also observed by Power et al. (1998). The capacity of *Calluna* biomass to incorporate N under current atmospheric inputs would also demonstrate that heathland vegetation in the Cantabrian Mountains is still N-limited (Pilkington et al., 2005b; Jones and Power, 2012; Southon et al., 2012). The soil microbial biomass pool is not an important N sink, only being able to incorporate scarce amounts of N inputs (~ 0.2%). N leaching losses in Cantabrian heathlands were negligible (~ 0.01% N inputs), and therefore, this demonstrates that they are still N-limited with the current rates of N inputs.

In addition to determining the distribution and fate of incoming N depositions within the heathland ecosystem, it would also be essential to assess how increasing N loads affect ecosystem functioning, and vegetation composition and structure. These mountainous heathlands are located in their southern-most distribution area (NW Spain), and it is therefore expected that they could respond differently to increasing N depositions compared to lowland heathlands (Meyer-Grünefeldt et al., 2016). It was observed that the main changes in the functioning of these Cantabrian heathlands occurred under long-term inputs (10 years) of high N load (56 kg N ha^{-1} yr^{-1}). Among these functioning changes, there was an increase in soil NH_4^+ content, which was associated with higher N content in plant tissues by increasing N uptake to support faster shoot growth rates (Pilkington et al., 2005b; Jones and Power, 2012; Phoenix et al., 2012; Southon et al., 2012). At the same time, the rise in *Calluna* shoot N content implied greater plant P demand due to nutrient imbalances associated to plant nutrient stoichiometry (Phoenix et al., 2003; Pilkington et al., 2005a). The higher demand of P was supplied by the stimulation of acid phosphatase enzymatic activity to mineralize organic P (Rowe et al., 2008; López-Poma and Bautista, 2014); and by the spreading of the root colonization by mycorrhizal fungi to facilitate the uptake of organic and inorganic P (Rowe et al., 2008; Jones and Power, 2012). The N- and P-enriched shoots would then be slowly incorporated into the litter layer, which would reflect the nutrient-enriched status of plant shoots (Pilkington et al., 2005b).

Higher N availability was reflected in the increased abundance of N-tolerant graminoid species, since they found an adequate opportunity to colonize high N availability environments (Calvo et al., 2005; Bobbink et al., 2010; Friedrich et al., 2011b). Regardless of N load, the cover of perennial graminoids was higher in young heathlands due to the colonization of opened canopy gaps (Barker et al., 2004; Friedrich et al., 2011b; Camac et al., 2013). By contrast, a strong decline in the abundance of N-sensitive cryptogam species due to N phytotoxicity effects was observed (Carroll et al., 1999;

Pearce and Van der Wal, 2002), being replaced by nitrophilous graminoids and forbs (Pilkington et al., 2007; Bähring et al., 2017). However, these effects are mainly manifested under long-term N inputs (10 years), displaying a great resistance to induced shifts from N inputs. In addition, the lack of significant changes in dominant woody species could be due to the resistant strategies they develop to respond to frequent environmental stresses (Calvo et al., 2002a, 2007; Camac et al., 2013), and to the resilient mechanisms to recover the pre-disturbed vegetation structure and composition after frequent perturbations (Calvo et al., 2002b). Other N-related effects in Cantabrian Mountains heathlands have been observed in *Calluna* vital rates (i.e., *Calluna* shoot growth and flowering), which positively respond to short-term N inputs (Lee and Caporn, 1998; Britton and Fisher, 2008; Cuesta et al., 2008; von Oheimb et al., 2010; Phoenix et al., 2012; Southon et al., 2012, 2013; Bähring et al., 2017). Nevertheless, they reduced this positive response under chronic N inputs. Besides, higher *Calluna* flowering and shoot N content was observed in young heathland stands.

The heathland life-cycle stage is also a noteworthy factor determining the impact of increasing N loads (Jones and Power, 2015; Meyer-Grünefeldt et al., 2015) on soil functioning and vegetation structure and composition. Thus, the vegetation growth phase should be considered in the establishment of N critical loads (Reynolds et al., 1998). For young and mature heathlands, the critical load for N deposition was established in the range of 10-20 kg N ha^{-1} yr^{-1} based on an increase in *Calluna* shoot N content and flowering at 14.6 kg N ha^{-1} yr^{-1}, which is in accordance with the current empirical range established for dry heathlands (10-20 kg N ha^{-1} yr^{-1}) by Bobbink and Hettelingh (2011). Thus, the potential reproductive of *Calluna* through changes in

Calluna phenology (no. flowers per shoot) could be an indicator to establish the N critical load regardless of heathland life-cycle stage.

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Conclusions

1. Bulk inorganic nitrogen depositions in the Cantabrian Mountains are within the range of 2.8-4.6 kg N ha^{-1} yr^{-1}, which are mainly deposited during the rainiest months. The nitrogen oxidized forms (NO_Y) prevail over reduced (NH_X) forms.

2. Heathlands dominated by *Calluna vulgaris* in the Cantabrian Mountains retain 72% of atmospheric nitrogen depositions in the short term (less than 1 year). The soil organic horizon represented the compartment with the greatest retention capacity. Negligible nitrogen leaching losses (< 0.01%) would demonstrate that this system still remains nitrogen-limited. In the long term (2 years), 95% of atmospheric nitrogen deposition was lost from the heathland ecosystem.

3. Increasing nitrogen inputs affect the functioning of *Calluna vulgaris* heathlands in the Cantabrian Mountains by increasing soil available NH_4^+, acid phosphatase enzyme activity, *Calluna vulgaris* shoot nitrogen and phosphorus contents, litter nitrogen content, as well as a greater *Calluna vulgaris* root colonization by ericoid mycorrhizae. These effects are exacerbated by chronic high nitrogen inputs (56 kg N ha^{-1} yr^{-1}; over 10 years). Heathlands in the mature phase display greater functional resistance in response to higher nitrogen availability.

4. Neither plant community composition nor plant species richness in *Calluna vulgaris* heathlands are significantly affected by the increase in nitrogen load, even under chronic high nitrogen inputs. However, annual and perennial herb species (graminoids and forbs) cover is positively affected by cumulative high nitrogen loads (56 kg N ha^{-1} yr^{-1}; 10 years). By contrast, cryptogam species cover, for instance, bryophytes and lichens, declined with chronic high nitrogen loads.

5. The effects of enhanced N loads on the heathland dominant species *Calluna vulgaris* consisted in an increase in its vital rates (shoot growth and flowering), and in shoot nitrogen and phosphorus contents.

6. In general, chronic and high nitrogen loads (56 kg N ha^{-1} yr^{-1}; 10 years) are able to stronger disrupt ecosystem functioning and vegetation structure of montane heathlands, mainly in their young phase.

7. For Cantabrian Mountains heathlands, the proposed nitrogen critical load is established in 14.6 kg N ha^{-1} yr^{-1} in both young and mature phases. The main indicators of these critical loads are those associated to the behaviour of the dominant species, *Calluna vulgaris*, such as the number of flowers per shoot and shoot nitrogen content.

Conclusiones

1. Las deposiciones inorgánicas de nitrógeno en la Cordillera Cantábrica varían entre 2,8 y 4,6 kg N / ha año, las cuales fueron más abundantes durante los meses con mayor precipitación. En general, las deposiciones de nitrógeno en formas oxidadas (NO_Y) son mayores que en formas reducidas (NH_X).

2. Los brezales dominados por *Calluna vulgaris* de la Cordillera Cantábrica retienen a corto plazo el 72% de las deposiciones atmosféricas de nitrógeno. El horizonte orgánico del suelo es el compartimento del ecosistema que presenta mayor capacidad de retención de nitrógeno. No se observan pérdidas importantes por lixiviación (< 0,01% de las deposiciones), lo que demuestra que el sistema sigue estando limitado por nitrógeno. A largo plazo (2 años) se pierde el 95% de las deposiciones que llegan a estos brezales.

3. El aumento de las cargas de nitrógeno afecta al funcionamiento de los brezales de *Calluna vulgaris* de la Cordillera Cantábrica mediante un incremento del contenido de NH_4^+ en el suelo, de la actividad enzimática fosfatasa ácida, del contenido en nitrógeno y fósforo en los brotes de *Calluna vulgaris,* del contenido en nitrógeno en la hojarasca, así como un aumento en el grado de colonización de las raíces de *Calluna vulgaris* por micorrizas ericoides. Estos efectos son mucho más pronunciados bajo situaciones de deposiciones crónicas (56 Kg N / ha año durante

10 años). Los brezales en fase madura muestran mayor resistencia funcional frente al aumento de las cargas de nitrógeno.

4. El aumento de las cargas de nitrógeno en los brezales de *Calluna vulgaris* no afecta significativamente ni a la composición ni a la riqueza de especies vegetales. Sin embargo, se ha observado un efecto positivo en la cobertura de las especies herbáceas anuales y perennes bajo cargas acumulativas de nitrógeno (56 Kg N / ha año durante 10 años). Por el contrario, la abundancia de criptógamas como líquenes y briófitos es afectada negativamente bajo deposiciones crónicas de nitrógeno.

5. Los efectos del aumento de las cargas de nitrógeno sobre la especie dominante en estos ecosistemas de brezal, *Calluna vulgaris*, se manifiestan mediante un incremento en sus rasgos vitales (número de flores por brote y longitud de los brotes), y también por un aumento en el contenido de nitrógeno y fósforo en los brotes.

6. En general, las cargas elevadas (56 kg N / ha año) y crónicas (10 años) de nitrógeno alteran de forma más pronunciada el funcionamiento y modifican más intensamente la estructura de la vegetación de los brezales de *Calluna vulgaris* de montaña, principalmente en fase joven.

7. Para los brezales de *Calluna vulgaris* de la Cordillera Cantábrica, la carga crítica para las deposiciones atmosféricas de nitrógeno está establecida en 14,6 kg N / ha año tanto para brezales jóvenes como maduros. Los indicadores para establecer esta carga crítica se basan en cambios en la especie dominante *Calluna vulgaris* tales como el número de flores por brote y el contenido en nitrógeno de los brotes.