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

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ABSTRACT

Ecosystems adapted to low nitrogen (N) conditions such as *Calluna*-heathlands are especially sensitive to enhanced atmospheric N deposition that affects many aspects of ecosystem functioning like nutrient cycling, soil properties and plant-microbial-enzyme relationships. 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 enzymatic activities, and plant root ericoid 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 a statistically 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. Our results also evidenced a considerable positive impact of chronic (10-year) high-N loading on soil extractable N-NH_4^+, acid phosphatase enzyme activity, *Calluna* root mycorrhizal colonization by ericoid fungi, *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 ericoid 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, is important for assessing the effects of added N, particularly at the heathlands' southern distribution limit.

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 at a global scale (Galloway et al., 2004), with slight differences between developing regions and industrialized ones (Vet et al., 2014). Airborne N loading 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 (Bähring et al., 2017; Phoenix et al., 2012). Moreover, chronic N loading 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; Fagúndez, 2013; Jones and Power, 2012; Meyer-Grünefeldt et al., 2016; Southon et al., 2012). Both field-scale surveys and N-manipulation experiments testing the effects of a variety of N-loading 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; Friedrich et al., 2011; Power et al., 2006; 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 loading is expected to aggravate the impact of N even at low input rates (Phoenix et al., 2012; Power et al., 2006).

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, flower-

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ing, tissue and litter chemistry or plant susceptibility to biotic (e.g., pathogen or pests) and abiotic (e.g., frost or drought) stressors] (Bähring et al., 2017; Jones and Power, 2012; Marcos et al., 2003; Meyer-Grünefeldt et al., 2016; Southon et al., 2013; Taboada et al., 2016). Elevated N inputs stimulate N mineralization rates (Phoenix et al., 2012), resulting in increased soil extractable N-NH_4^+ and N-NO_3^- (Boot et al., 2016; Song et al., 2017; Southon et al., 2013). This enhanced soil N availability may cause either an increase (Du et al., 2014; Haugwitz et al., 2011) 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 (Contosta et al., 2015; Ramírez et al., 2012; Zhu et al., 2015), and the ericoid mycorrhizal (ERM) fungal community associated with *Calluna* in nutrient-poor environments (Caporn et al., 1995; Yesmin et al., 1996). Since soil microorganisms are considered the primary sources of soil enzymes, and these are involved in nutrient metabolism and decomposition processes (Fatemi et al., 2016; Ramírez et al., 2012; Sinsabaugh and Follstad, 2012; Song et al., 2017; Zhu et al., 2015), 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; Jian et al., 2016; Ochoa-Hueso et al., 2011, 2014). These variations very likely affect the storage, turnover and uptake of soil nutrients (Cenini et al., 2016; Jones and Power, 2012). 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 (Calvo et al., 2007; Jones and Power, 2012; Pilkington et al., 2005b; Rowe et al., 2008; 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 (Fagúndez, 2013; Härdtle et al., 2006, 2009), 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).

In contrast to north-western (e.g., Phoenix et al., 2012; Southon et al., 2012) and central European (e.g., Bähring et al., 2017; de Vries et al., 2009; Friedrich et al., 2011) *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 loading 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 loading 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; Southon et al., 2013; von Oheimb et al., 2010); (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 (Haugwitz et al., 2011; Power et al., 2006), 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 loading (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).

2. Material and methods

2.1. Study area

The study area is located on the southern slope of the Cantabrian mountain 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 to 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.

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 × 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 experimental plots were randomly assigned to the N treatments at each study site.

During the experiment, infestations of *Calluna* plants by the larvae of 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), but only ca. 0.5% of the heathland area was infested (see Taboada et al., 2016 for further information). In the 2008 outbreak event, only the chronic high N treatment (N56) mature plots were severely defoliated, but the consumed plants were not killed and regenerated the next growing season following the beetle's attack. In the 2015 outbreak event, both mature and young N56 plots were marginally affected by the beetle's defoliation. 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.

2.3. Sampling methods

We collected three soil samples (topsoil, 0–5 cm below the litter layer) in each 2 m × 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 ericoid mycorrhizal colonization in August 2015. Fine roots were manually isolated using a 400 × 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 × 5 cm litter layer (i.e., dead plant leaves fell to the ground) samples from each 2 m × 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.

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 2 M 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 (1965) method. 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, 1982; Tabatabai and Bremner, 1969). 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 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 ac-

cording 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 ericoid mycorrhizae using the intersection method by McGonigle et al. (1990), with a 400 × 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.

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 ericoid mycorrhizae 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 ericoid mycorrhizae 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. Results

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. 1; Table 1). However, there were no significant differences for soil extractable N-NO_3^- (Fig. 1; Table 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. 1; Table 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 1).

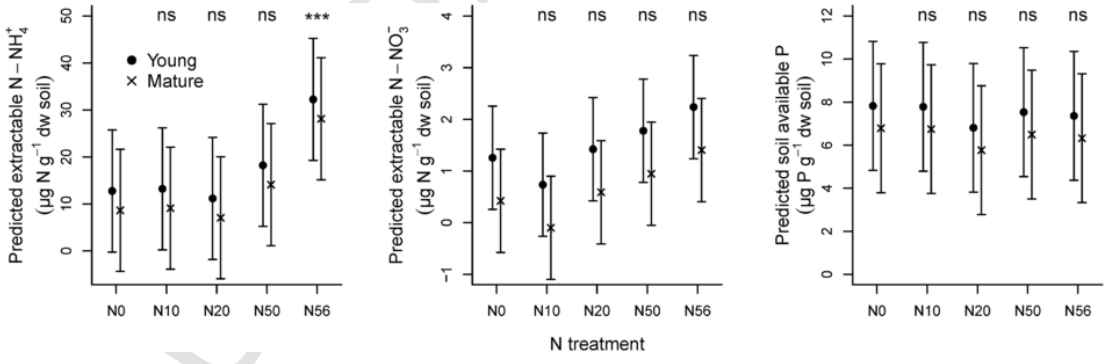

Fig. 1. Model predicted values (mean ± 95% confidence intervals) of soil nutrient content variables in relation to stand age (young vs. mature) and the five N treatments (N0, N10, N20, N50, and N56): extractable N-NH_4^+ (µg N g^{-1} dw soil), extractable N-NO_3^- (µg N g^{-1} dw soil), and available 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 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. The interaction term (age × 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 and the sampling years) are given. Df = degrees of freedom. Significant p-values are in bold face.

Response variable	Fixed effects				Random effects		
	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-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-NO_3^-	Age	1	4.17	**0.043**	Site	8.5×10^{-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 nutrient contents							
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

3.2. Enzymatic activities

Acid phosphatase enzyme activity significantly increased in response to N addition, particularly in the N56 treatment, and to a lesser extent in the N10 treatment (Fig. 2; Table 1). However, there were no significant differences in acid phosphatase activity related to heathland age. We found significant differences in urease enzyme activity after the addition of N in the N10 treatment (Fig. 2; Table 1); but no significant differences in relation to heathland age. β-Glucosidase enzyme activity did not show significant changes in relation to N addition and heathland age (Fig. 2; Table 1).

3.3. Soil microbial biomass

Soil microbial biomass C and N did not show any significant changes in response to the addition of N (Fig. 3; Table 1). Microbial biomass N content was, however, significantly higher in young heathlands than in mature ones.

3.4. Calluna root mycorrhizal colonization

The percentage of *Calluna* roots colonized by ericoid mycorrhizae was significantly higher in the N56 treatment (Fig. 4; Table 2). Moreover, the responses of mycorrhizal colonization to the N treatments were age-related, shown by the significant 'age × 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.

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. 5; Table 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. 5; Table 3). Besides, higher litter N content was found in young heathlands than in mature ones. A significant 'age × N treatment' interaction was found for litter P content (Fig. 5; Table 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. 5; Table 3).

4. Discussion

4.1. Time- and dose-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 semi-arid 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 loading. 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,

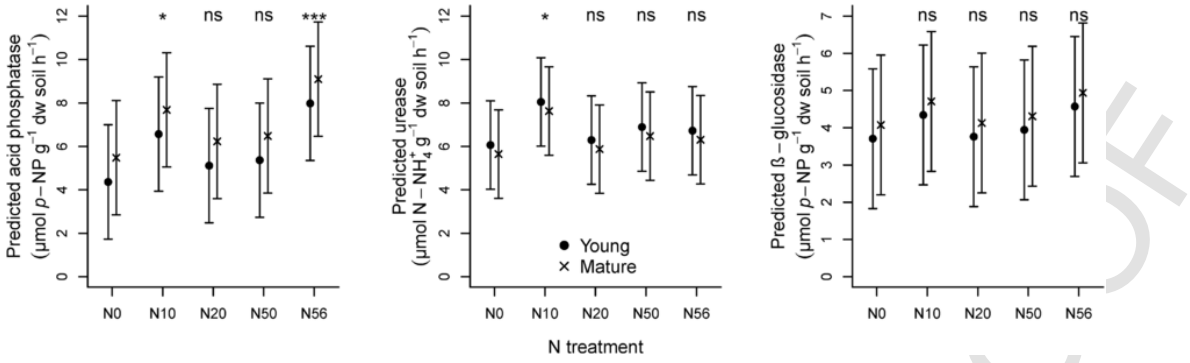

Fig. 2. Model predicted values (mean ± 95% confidence intervals) of soil enzymatic activities in relation to stand age and the N treatments: acid phosphatase (μmol *p*-NP g^{-1} dw soil h^{-1}), urease (μmol N-NH_4^+ g^{-1} dw soil h^{-1}), and β-glucosidase (μmol *p*-NP g^{-1} dw soil h^{-1}). Significance 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).

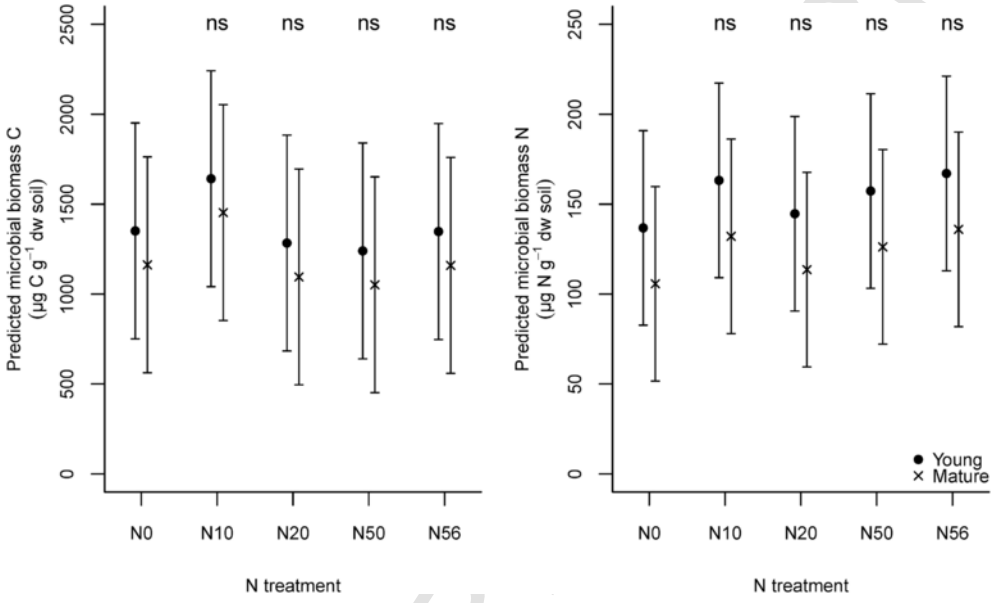

Fig. 3. Model predicted values (mean ± 95% confidence intervals) of the nutrient contents of soil microbial biomass in relation to stand age and the N treatments: C (μg C g^{-1} dw soil), and N (μg N g^{-1} dw soil). Significance levels of the difference between each N fertilization treatment (N10, N20, 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).

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 loading. 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 > 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 stock 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 enzyme activities, determined by the levels of metabolic nutrient demands (Jian et al., 2016; Ochoa-Hueso et al., 2011; Sinsabaugh and Follstad, 2012; 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 (i.e., higher N availability), resulting in P deficiency for plants and soil microbes in low available P status of heathland soils (Ochoa-Hueso et al., 2014; Phoenix et al., 2003; Pilkington et al., 2005a). This nutritional imbalance could be compensated for by the microbial

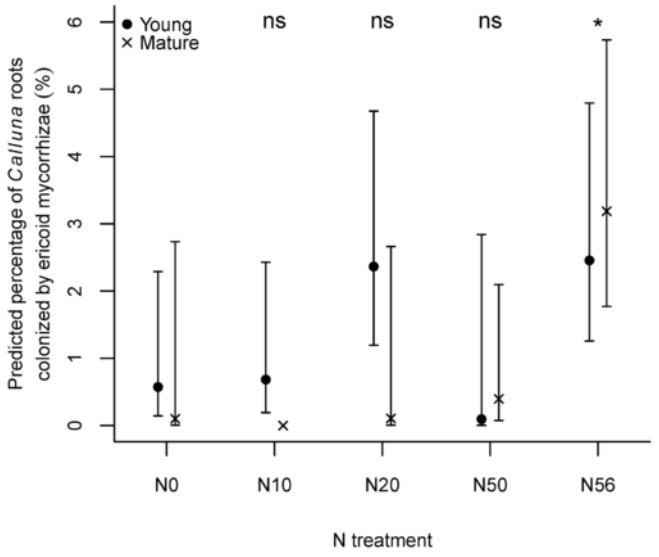

Fig. 4. Model predicted values (mean ± 95% confidence intervals) of *Calluna* mycorrhizal colonization (% roots colonized by ericoid mycorrhizae) in relation to stand age and the N treatments. Significance levels of the difference between each N fertilization treatment (N10, N20, N50, and N56) and the 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 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 by ericoid fungi. 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 ericoid mycorrhizae	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**

and plant excretion of phosphatase enzymes required for the mineralization of organic P (López-Poma and Bautista, 2014; Pilkington et al., 2005a; Rowe et al., 2008); since phosphatase activity can be modulated through enzyme excretion or activity inhibition depending on soil P availability (Johnson et al., 1999; López-Poma and Bautista, 2014; Phoenix et al., 2003).

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; Fatemi et al., 2016; Sinsabaugh and Follstad, 2012; 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 (Cenini et al., 2016; López-Poma and Bautista, 2014), 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 (Calvo-Fernández et al., 2015; Hagedorn et al., 2010). 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 (Contosta et al., 2015; Rinnan et al., 2007).

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 (Jones and Power, 2012; Pilkington et al., 2005b; 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; Friedrich et al., 2011; von Oheimb et al., 2010). 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 taken up by the plants or incorporated into the soil microbial biomass to satisfy their enhanced P demands in response to the addition of N (Friedrich et al., 2011; Johnson et al., 1998, 1999; Jones and Power, 2015; Rowe et al., 2008); or even immobilized by iron and aluminium (hydr)oxides in acid soils (Kooijman et al., 1998). Particularly, the enhanced P demand of *Calluna* plants in the chronic high N treatment (N56) in low available P status of heathland soils 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 (López-Poma and Bautista, 2014; Pilkington et al., 2005a; Rowe et al., 2008); and by (2) the observed increase in the degree of *Calluna* root mycorrhizal colonization by ericoid fungi that might enhance plant nutrient uptake from the widely extended hyphae network (Díaz et al., 2006; Jones and Power, 2012; Rowe et al., 2008), including the nutrient uptake of organic forms (Johnson et al., 2005). Besides, ericoid mycorrhizal fungi are able to produce and release acid phosphatase enzyme to provide inorganic P (Cairney and Burke, 1998). By contrast, other heathland studies showed that increasing N loadings tended to decrease the ericoid mycorrhizal colonization in *Calluna* roots due to the little benefit of mycorrhizal fungi in non-limited nutrient conditions (Yesmin et al., 1996). Moreover, a decrease in root mycorrhizal colonization with increasing N inputs was also observed by Camenzind et al. (2016) in an N-fertilization experiment in montane forests, pointing out that the response of mycorrhizal fungi to N loading depends on the soil fertility status previous to fertilization.

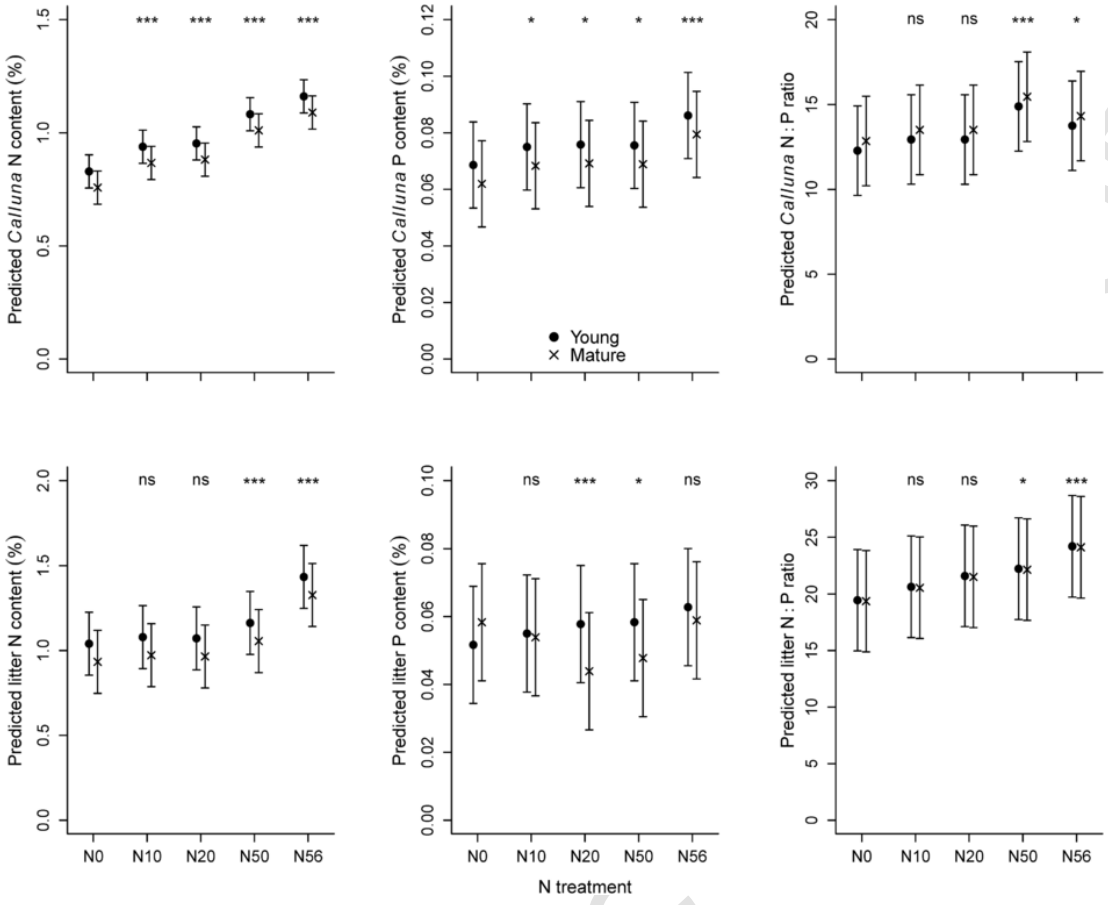

Fig. 5. Model predicted values (mean ± 95% confidence intervals) of *Calluna* shoot and litter nutrient contents in relation to stand age and the N treatments: N (%), P (%), and N:P ratio. Significance levels of the difference between each N fertilization treatment (N10, N20, N50, and N56) and the control treatment (N0) are indicated by *** (0.001 > p), ** (0.01 > p > 0.001), * (0.05 > p > 0.01), and ns (p > 0.05).

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 $N\text{-}NO_3^-$ 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-loading. 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 (Díaz et al., 2006; Read and Pérez-Moreno, 2003). 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 (Díaz et al., 2006; Johnson et al., 1999). Besides, higher aboveground biomass of mature stands could transfer greater amounts of photosynthesized-carbohydrates to ericoid mycorrhizal fungi for extending the mycelial network and increasing the 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 (Gimingham, 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.

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 loading (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 (Calvo et al., 2005; Marcos et al., 2003) or require longer time periods of N inputs before the deleterious effects of N loading 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

Table 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. The interaction term (age × 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 and the sampling years) are given. Df = degrees of freedom. Significant p-values are in bold face.

Response variable	Fixed effects				Random effects		
	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

causative factors for this lack of response to the added N (Hagedorn et al., 2010). Furthermore, the apparent ability of montane *Calluna* plants to withstand disturbance factors (i.e., insect defoliation, drought and frost; see Section 2.2 'Experimental design'), suggests that these marginal heathlands may be quite resilient to N loading. 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 long-term research is needed to fully understand the effects of chronic N deposition and its interactions with other episodic biotic and abiotic stressors (like insect pest outbreaks; Taboada et al., 2016) and global environmental change factors (Meyer-Grünefeldt et al., 2016) on the functioning of montane heathlands.

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 loading 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 loading. *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.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.scitotenv.2017.08.307. These data include the Google map of the most important areas described in this article.

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