



Resistance of soil bacterial communities from montane heathland ecosystems in the Cantabrian mountains (NW Spain) to a gradient of experimental nitrogen deposition

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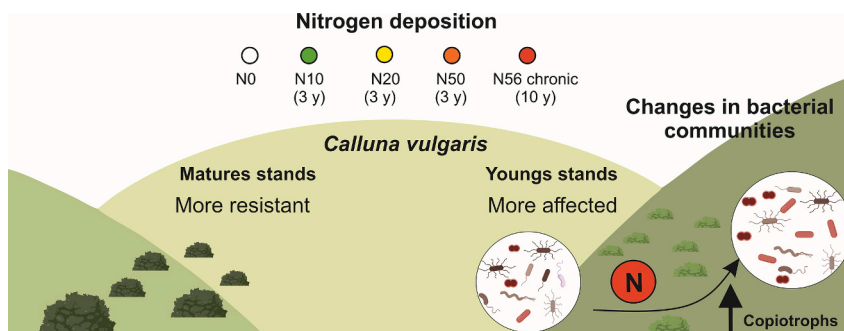
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HIGHLIGHTS

- Analysis of the effect of N deposition on soil bacterial communities in heathlands
- The diversity of bacterial communities was resistant to N addition in mature stands.
- Long-term chronic N addition increased bacterial diversity in young heathlands.
- Shifts mediated by changes in dominance of oligotrophic/copiotrophic taxa

GRAPHICAL ABSTRACT



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ABSTRACT

Elevated atmospheric nitrogen (N) deposition on terrestrial ecosystems has become one of the most important drivers of microbial diversity loss on a global scale, and has been reported to alter the soil function of nutrient-poor, montane *Calluna vulgaris* heathlands in the context of global change. In this work we analyze for the first time the shifts of bacterial communities in response to experimental addition of N in *Calluna* heathlands as a simulation of atmospheric deposition. Specifically, we evaluated the effects of five N addition 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 the resistance of soil bacterial communities as determined by changes in their composition and alpha and beta diversities. The study was conducted in montane *Calluna* heathlands at different development stages (young and mature phases) in the southern side of the Cantabrian Mountains (NW Spain). Our results evidenced a substantial increase of long-term (10-years) N inputs on soil extractable N-NH₄⁺, particularly in young *Calluna* stands. The alpha diversity of soil bacterial communities in mature *Calluna* stands did not show a significant response to experimental N addition, whereas it was significantly higher under long-term chronic N addition (56 kg N ha⁻¹ yr⁻¹ for 10-years) in young *Calluna* stands. These bacterial community shifts are mainly attributable to a decrease in the dominance of

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Acidobacteria phylum, the most representative in montane *Calluna* ecosystems, in favor of copiotrophic taxa such as Actinobacteria or Proteobacteria phyla, favored under increased N availability. Future research should investigate what specific ecosystem functions performed by soil bacterial communities may be sensitive to increased nitrogen depositions, which may have substantial implications for the understanding of montane *Calluna* ecosystems' stability.

1. Introduction

Soil bacteria are among the most diverse and abundant organisms on Earth (Ramírez et al., 2014) and play a key role in regulating nutrient cycles and plant productivity (Delgado-Baquerizo et al., 2018). Climate (Maestre et al., 2015; Zhou et al., 2016), nutrient availability (Leff et al., 2015), vegetation type (Prober et al., 2015) and pH (Lauber et al., 2009), among others, can influence the composition of soil bacterial communities. Microbial community structure may be indicative of ecosystem processes related to energy, and nutrients' fluxes (Cardinale et al., 2012; Graham et al., 2016). Understanding the forces controlling biological diversity and, in turn, the role of biological diversity in ecosystem functions is of utmost importance for predicting the consequences of increasingly frequent ecological disturbances in the context of global change.

Since the 1970s, anthropogenic activities have contributed to an increase in reactive nitrogen (N) in the atmosphere, and the N deposition on terrestrial ecosystems has become one of the most significant determinants of microbial diversity loss on a global scale (Galloway et al., 2014). Contosta et al. (2015) indicate that aboveground N additions may cause changes in microbial biomass and bacterial community composition. Bacterial communities can resist atmospheric N inputs for years or decades as a function of resource availability and thus ecosystem type and environmental conditions (Contosta et al., 2015; Zhu et al., 2015). Therefore, long-term experiments are imperative to detect bacterial community shifts with potential implications on soil carbon (C) and N cycling, in accordance with previous research (Lipson et al., 1999; Waldrop and Firestone, 2006; Power et al., 2006; Du et al., 2014; Boot et al., 2016; Calvo-Fernández et al., 2018).

According to the European Habitats Directive 92/43/EEC, *Calluna vulgaris* (L.) Hull heathlands are one of the most representative protected ecosystems in northwestern Europe mountains. These ecosystems are adapted to conditions of low N concentrations (Calvo-Fernández et al., 2015). *Calluna vulgaris* (hereafter *Calluna*) heathlands are very sensitive to N deposition (Southon et al., 2012; Meyer-Grünefeldt et al., 2015) causing substantial changes in plant composition, structure and functioning, even threatening their persistence in Europe (Calvo et al., 2005, 2007; Friedrich et al., 2011; Fagúndez, 2013). For example, it has been reported that experimental N addition may (i) promote changes in plant growth, including heighten *Calluna* shoot length (Taboada et al., 2018) and drought sensitivity (Meyer-Grünefeldt et al., 2016), (ii) reduce plant species diversity (Southon et al., 2013), (iii) increase the depth of the litter layer and the decomposition rates of soil organic matter (Jones and Power, 2012; Villalón, 2014), or (iv) disrupt trophic interactions involving *Calluna* herbivores (Taboada et al., 2016).

Experimental studies with controlled experimental N addition have shown that, even at high fertilization rates, European heathlands can retain 60–90 % of the Supplied N. (Pilkington et al., 2005). The bacterial biomass in the soil organic horizon easily assimilates N from atmospheric sources, accounting for approximately two-thirds of the total N pool in the ecosystem (Phoenix et al., 2012), reducing the microbial C:N ratio (Perakis et al., 2005) and increasing microbial activity (Power et al., 2006). Soils of *Calluna* heathlands are also capable of immobilizing, mainly in the organic horizon, about 47 % of N deposited from the atmosphere (Calvo-Fernández et al., 2015). Furthermore, Tye et al. (2005) determined that the soil bacterial community of the organic layer immobilizes 50–70 % of the ¹⁵N recovered in the humic fraction. The maturity degree of *Calluna* heathlands may also modulate the effects of

atmospheric N deposition on the ecosystem nitrogen cycling, decomposition rates and microbial activity (Barker et al., 2004; Jones and Power, 2015; Wendling et al., 2016).

Calvo-Fernández et al. (2018) and Taboada et al. (2018) analyzed the effect of N addition simulating atmospheric deposition on the structure and functioning of montane *Calluna* heathland ecosystems in three areas of Northern Spain. Their results showed that the composition of vegetation functional groups, species diversity and productivity of these *Calluna* heathlands at the southernmost limit of their distribution range were moderately resistant to cumulative N addition. They only reported an increase in soil extractable ammonium (N-NH₄⁺), acid phosphatase enzymatic activity and colonization of *Calluna* roots by ericoid mycorrhizae, although these changes were only detected in long-term plots subjected to chronic N inputs for 10-years. These findings in *Calluna* heathlands located at rear-edge limits should be complemented with in-depth analyses of the capacity of soil bacterial communities to resist disturbance produced by cumulative nitrogen inputs for improving the proactive management of *Calluna* heathlands threatened by global change feedbacks (Delgado-Baquerizo et al., 2018).

In the global change context, previous studies have analyzed the effect of several ecological disturbances and restoration activities on soil microbial communities in *Calluna* heathlands. Tibbett et al. (2019) analyzed how long-term experimental acidification with sulfur affected soil microbial biodiversity and function in a heathland restoration scheme in southern England, demonstrating a significant reduction in soil microbiota in these acidified soils. The effects of experimental long-term drought and warming on soil bacterial and fungal communities were examined in several upland heathlands in North Wales to unravel ecosystem responses to climate change (Seaton et al., 2022). They found that changes in the microbial communities were more pronounced after longer periods of climate manipulation. Andresen et al. (2014) and Reinsch et al. (2014) analyzed the combined effects of elevated temperature and atmospheric CO₂ concentration, together with extended summer drought, on the responses of microbial community structure and diversity, as well as the shifts in substrate utilization by soil microorganisms in several heathland experimental field sites in Denmark. Drought scenarios favored the rhizosphere bacterial community, while elevated temperature reduced gram-negative abundance and altered the actinomycetes community composition. However, to the best of our knowledge, studies concerning the effects of increased deposition of atmospheric N on soil microbial communities in *Calluna* heathlands, particularly those located at the southernmost limit of their range of distribution, are completely absent up to date. Furthermore, the effect of the *Calluna* ecosystem development stage on these feedbacks has remained unexplored.

Accordingly, in this work we analyze for the first time the shifts of bacterial communities in response to the disturbance caused by the experimental addition of N as a simulation of atmospheric deposition in the context of global change. Three different behaviors are possible: (i) change in the diversity of the bacterial communities with variations in the abundance of some bacterial taxa, proportional to the intensity of the disturbance; (ii) lack of any type of change, showing total resistance of bacterial communities, i.e. high ability to withstand disturbance relative to the control situation (e.g. Huang et al., 2020; Wang et al., 2020); and (iii) partial resistance in which the bacterial community would change once a certain intensity of the disturbance is exceeded. Therefore, the objective of this work is to determine the resistance of soil bacterial communities as determined by changes in the soil environment

mediated by N deposition simulations on nutrient-poor, montane *Calluna* heathlands located at the southern European limit of their distribution. Specifically, we tried to unravel the effects of increasing N fertilization rates on soil chemical properties and the associated impacts in the composition and alpha and beta diversity of soil bacterial communities under different *Calluna* development stages.

2. Materials and methods

2.1. Study site description and experimental design

The study was conducted in a *Calluna*-heathland site (1660 m a.s.l., 43°02'N, 5°24'W, 24 ha) with a dominant north slope aspect and steep slopes, located on the Cantabrian Mountains (NW Spain). The climate of the site is Eurosiberian with a mean annual temperature and annual precipitation of 5.5 °C and 1645 mm, respectively. Precipitation is unevenly distributed throughout the year, with 2–3 months of summer drought (Calvo-Fernández et al., 2017), and occurs mainly in the form of snow in winter, as well as in late-autumn and early-spring. Based on the EMEP and CHIMERE models (Bobbink et al., 2010; Hall et al., 2015), total N deposition in the site falls between 7.5 and 15 kg N ha⁻¹ yr⁻¹ (García-Gómez et al., 2014), which is below or within the range of the lowest critical load value that may threaten the survival of European dry *Calluna* heathlands (i.e. 10–20 kg N ha⁻¹ yr⁻¹). Soils are classified as Umbrisols, with high acidity (pH = 4.17 ± 0.22), sandy texture and low fertility. A thin litter layer (< 1 cm depth) arranged in discontinuous patches characterizes young *Calluna* stands, whereas a homogeneous and thicker litter layer (2–3 cm) covers the soil in mature stands. The site is dominated by *Calluna vulgaris*, with a cover higher than 75 %, and a mean height of approximately 20 cm in young heathlands and 50 cm in mature ones. The main accompanying species are *Erica tetralix* L. and *Vaccinium myrtillus* L.

We established 30 square plots of 2 m × 2 m in the site. The manipulative experiment consisted of a randomized assignment of five N addition treatments in each *Calluna* stand (i.e. 3 replicates per N treatment and *Calluna* age class). The young plots were located in *Calluna* stands affected in 2005 by a prescribed fire, and the mature plots were located in *Calluna* stands that showed signs of degradation after 30–40 years of abandonment. These time ranges correspond approximately to building and mature phases in heathlands (Gimingham, 1972). To explore the effects of N disturbance intensity on the resistance of soil bacterial communities, the treatment consisted in the monthly addition by hand during 3-years (June–November from 2013 to 2015) of ammonium nitrate granules (27 % NH₄NO₃ purity) in 4 different doses of 0, 10, 20 and 50 kg N ha⁻¹ yr⁻¹ besides background atmospheric N deposition (N0, control; N10, low N load; N20, medium N load; and N50, high N load, respectively). Additionally, plots subjected to a chronic treatment of 56 kg N ha⁻¹ yr⁻¹ for 10-years (N56, chronic high N load added monthly in May–October from 2005 to 2015) were also considered. This load is equivalent to the highest expected atmospheric N input by 2050 for southern European countries (Galloway et al., 2004; Boot et al., 2016), and may help to evaluate what would happen if we pushed *Calluna* heathlands into N saturation as experienced in other ecosystems (Boot et al., 2016). In addition, the difference between the highest accumulated N doses (N input × addition period) and background deposition in our study site is consistent with that of previous research in heathland ecosystems in Europe (Phoenix et al., 2012), as well as in other ecosystems worldwide (Moore et al., 2021). A similar experimental design has been widely implemented in previous studies (e.g. Taboada et al., 2016, 2018; Calvo-Fernández et al., 2018). Indeed, accumulated N doses are commonly applied where long-term datasets are available for evaluating responses in terms of cumulative N deposition, which may provide integrated insights into how ecosystem changes may develop over time under enhanced deposition rates, thereby identifying sensitivity to long-term inputs (Phoenix et al., 2012). Moreover, this approach (i) already integrates the time dimension, (ii)

allows the comparison of ecosystem responses between treated plots for different time periods, and (iii) improves the understanding of the changes over decadal timescales associated with chronic N deposition (Phoenix et al., 2012). Conversely, the use of high N loadings in a single addition to simulate long-term N deposition effects may lead to the overestimation of ecosystem impacts (Pearce and van der Wal, 2008).

2.2. Soil sampling and analyses

Three soil samples were randomly collected from each plot (topsoil, 0–5 cm below the litter layer) in September 2015. The samples were pooled in a composite soil sample per plot. The first soil fraction per sample was stored in a polyethylene bag and frozen at –20 °C until DNA extraction in the laboratory and soil analyses. N-NH₄⁺ (mg kg⁻¹) and N-NO₃⁻ (mg kg⁻¹) were extracted with 2 M KCl at 1:10 soil-extractant ratio (Keeney and Nelson, 1982), and subsequently measured by distillation with a micro-Kjeldahl automatic analyzer (Bremner and Mulvaney, 1982). The second soil fraction was air-dried in the laboratory for soil pH, total N, soil organic carbon (SOC), and available phosphorous (P) analyses. Soil pH was determined potentiometrically at a 1:2.5 ratio in H₂O. The Kjeldahl procedure was used to determine soil total N (%) (Bremner and Mulvaney, 1982). Soil organic C (%) was determined using the Walkley-Black dichromate wet oxidation method with potassium dichromate (Nelson and Sommers, 1982). We determined the soil available P (mg kg⁻¹) following the Bray-Kurtz method (Kalra and Maynard, 1991).

2.3. DNA extraction and Illumina sequencing

The Power Soil Pro DNA isolation kit (Quiagen Inc.) was used to extract the DNA from 0.25 g soil of each sample following the manufacturer's protocols. We spectrophotometrically determined DNA concentration and quality using a Nanodrop instrument (Thermo Scientific) and a Qubit 2.0 fluorometer (Invitrogen). The 515F (5'-GTGCCAGCMGCCGCGTAA) and 806R (5'-GGACTACHVGGGTWCTAAT) primers (Caporaso et al., 2012) were used to amplify the prokaryotic (bacterial and archaeal) V4 region of the 16S small subunit (SSU) rRNA. These primers were chosen to maximize the global coverage of Bacteria and Archaea while also providing suitable products for sequencing with Illumina platforms (Parada et al., 2016; Wasimuddin et al., 2020). The barcoded PCR libraries from each sample were quantified by real-time PCR in LightCycler 480 (Roche), pooled with equimolar concentrations and then sequenced by paired-end sequencing (250 × 2) in Illumina Miseq platform.

2.4. Sequence processing

Bioinformatic processing of raw sequences was performed using vsearch 2.17 (Rognes et al., 2016) tools. After oligos trimming, paired end sequences were merged and screened to remove low-quality and out range length (225 to 280 pb) reads with vsearch. Sequences were clustered in OTUs at 97 % identity level using cluster_size function of vsearch. Taxonomic assignment of each OTUs was obtained using the RDP database training set 18 and classifier tool v.2.13 (Wang et al., 2007).

Unique sequences were determined with vsearch and aligned using Infernal (Nawrocki and Eddy, 2013). A neighbor-joining tree was constructed on MEGA7 (Kumar et al., 2016) using the Kimura-2p evolutionary model. The phylogenetic tree and the table of frequencies of each OTU were constructed with vsearch.

The estimated alpha diversity metrics comprised (i) the rarefied bacterial richness and (ii) the Shannon's diversity index. We computed four beta diversity metrics, including ecological classic (i) Chao (qualitative) and (ii) Bray-Curtis (quantitative) indices, as well as (iii) unweighted UniFrac and (iv) weighted UniFrac phylogenetic metrics (Lozupone and Knight, 2005). The distance between pairs of samples

was explored using a principal coordinate analysis (PCoA) based on beta diversity indices among the heathland ecosystem age and N fertilization treatment levels.

The vegan (Oksanen et al., 2018), APE (Paradis et al., 2004) and GUniFrac (Chen et al., 2022) packages were used to estimate alpha and beta diversities in R (R Core Team, 2021).

2.5. Statistical analysis

Generalized linear models (GLMs) were fitted to evaluate the effect of heathland ecosystem age and N fertilization on the analyzed soil physicochemical and biological properties. The dependent variables were: (i) soil pH, (ii) soil extractable N-NH₄⁺, (iii) soil extractable N-NO₃⁻, (iv) soil total N, (v) soil organic C, and (vi) soil available P. The positive-only dependent variables were fitted using a Gamma error distribution with a log-link function, while the rest of the dependent variables were fitted using a Gaussian error distribution with an identity link function following Fernández-García et al. (2019). The predictors were ecosystem age (young and mature), the N treatment (N0, N10, N20, N50, and N56), and their interaction. We only retained the interaction term when it was significant. A Tukey HSD post-hoc test was used to assess statistical differences between each level of the N treatment factor. Statistical significance was considered at the 0.05 level. The same approach was followed to test for differences in the alpha diversity of the bacterial communities (dependent variables) between levels of heathland ecosystem age and N fertilization treatments (predictors). According to Fernández-Guisuraga et al. (2022), the GLM of the Shannon's diversity index was fitted assuming a Gaussian error distribution with an identity link function. To account for overdispersion, a quasi-Poisson (log link function) error distribution was used to fit the rarefied bacterial richness model.

The effect size for the contrast between N loading doses (N10, N20, N50, and N56) and the control was calculated for the frequencies of the most representative bacterial taxa (accounting for ≥1 % of the reads in the whole sample set) was calculated using the log response ratio (lnRR) assuming non-dependent samples across treatments, i.e. independent-groups design (Hedges et al., 1999; Lajeunesse, 2011). lnRR effect size metric has been extensively used in previous ecological research investigating the effect of manipulative experiments on soil microbial abundance (e.g. Zeng et al., 2016; Mushinski et al., 2019), providing a direct ecological interpretation of the treatment effect because the metric scales directly to the strength of the effect (Coleman, 2012; Lucas-Borja et al., 2022). A negative lnRR value for a N dose level indicates that the frequency of the bacterial taxa decreases compared to the control, while a positive value indicates that the abundance is higher than in the control. The N treatment significance was evaluated by means of the 95 %-confidence interval (CI₉₅) of lnRR using the Gaussian distribution (Lucas-Borja et al., 2022). If the CI₉₅ does not overlap with zero, the effect size of the N treatment is statistically significant (Luo et al., 2006).

All analyses were conducted in R (R Core Team, 2021).

3. Results and discussion

3.1. Soil chemical properties

The effect of N addition on soil chemical properties showed a stationary response at the age of the heathlands (i.e. no significant interaction between heathland ecosystem age and N fertilization treatment; Table 1). The content of soil extractable N-NH₄⁺ was significantly higher in the N56 treatment than in N0, N10 and N20 treatments (Table 1 and Fig. 1), but there were no significant differences in soil extractable N-NH₄⁺ between the N50 and any other dose, which shows that long-term N inputs play a fundamental role in *Calluna* heathlands. Conversely, soil extractable N-NO₃⁻ showed no significant differences between different levels of N addition, but its content was significantly higher in young than in mature heathlands (Table 1 and Fig. 1). Significantly higher soil

Table 1

Generalized linear models (GLMs) outputs for the effects of heathland ecosystem age and N fertilization treatment on the analyzed soil chemical properties. The interaction term only was retained in the model when significant. Significant p-values are marked in bold. Significance levels are represented by *** (*p*-value < 0.001), ** (*p*-value < 0.01), * (*p*-value < 0.05), and "ns" (*p*-value > 0.05).

Dependent variable	Predictor	Df	F	p-Value (>F)	
pH	Age	1	1.240	0.276	ns
	N treatment	4	0.882	0.490	ns
Extractable N-NH ₄ ⁺	Age	1	4.570	0.043	*
	N treatment	4	6.572	0.001	**
Extractable N-NO ₃ ⁻	Age	1	4.395	0.047	*
	N treatment	4	1.210	0.332	ns
Total N	Age	1	5.506	0.028	*
	N treatment	4	0.183	0.945	ns
Organic C	Age	1	4.618	0.043	*
	N treatment	4	0.226	0.921	ns
Available P	Age	1	0.081	0.778	ns
	N treatment	4	0.295	0.876	ns

total N and organic C values were registered in mature heathlands than in young ones, but N treatment effect was not significant (Table 1 and Fig. 1). Similarly, no significant differences were found for soil pH and available P with regard to the N treatments and heathland maturity levels (Table 1).

These results were consistent with previous research reporting an increase in extractable N-NH₄⁺ under high experimental N inputs simulating atmospheric N deposition in *Calluna* heathlands (13-years, >40 kg N ha⁻¹ yr⁻¹: Phoenix et al., 2012; 9-years, 56 kg N ha⁻¹ yr⁻¹: Calvo-Fernández et al., 2018), as well as in other ecosystems worldwide (e.g. Ochoa-Hueso et al., 2013; Song et al., 2017; Heindel et al., 2022; Sun et al., 2022). Particularly, the low soil pH values (pH = 4.17 ± 0.22), the harsh environmental conditions in the Cantabrian marginal montane heathlands studied here, and thus the expected low net nitrification rates (Stevens et al., 2011), may be responsible for (i) the high accumulation of soil extractable N-NH₄⁺, particularly under increased N addition, and (ii) the absence of a significant response in the extractable N-NO₃⁻. The reported increase in soil extractable N-NH₄⁺ only in the chronic N treatment agrees with previous research in upland *Calluna* heathlands (Phoenix et al., 2012; Calvo-Fernández et al., 2018). Also, the absence of a significant response in total N, available P and soil organic C pools by N addition could be mediated by the slow transfer rates between soil pools in response to increased N availability and the short periods of microbial activity in montane *Calluna* heathlands (De Vries et al., 2009; Ochoa-Hueso et al., 2014; Calvo-Fernández et al., 2018).

3.2. Composition of bacterial communities

A total of usable 2,153,029 reads were obtained from the 30 bacterial communities analyzed (5 N treatments × 2 ecosystem age × 3 replicates). Clustering allowed us to distinguish 68,604 phylotypes or OTUs. Rare OTUs with <100 reads in the communities were not considered for subsequent analyses. We thus retained the 6278 most frequent OTUs, which accounted for 94.9 % of the reads (2,043,857). The reads included in the analysis were distributed among the samples in a range between 41,827 and 111,358, with the mean equal to 68,129.

Twenty-one Bacteria and four Archaea phyla have been found in these heathlands, but only six of them showed higher frequency than 1 %. Acidobacteria, accounting for 36.42 % of all reads in the samples, was the most represented phylum over all treatments except in N56 (chronic N addition for 10 years), where Proteobacteria or Actinobacteria dominate depending on *Calluna* ecosystem maturity (Fig. 2). These two phyla (Proteobacteria and Actinobacteria) were also frequent in *Calluna* heathlands, with 17.55 % and 14.94 % of the reads, respectively. Proteobacteria showed higher frequency than Actinobacteria at most levels of ecosystem age and N addition. However, Actinobacteria

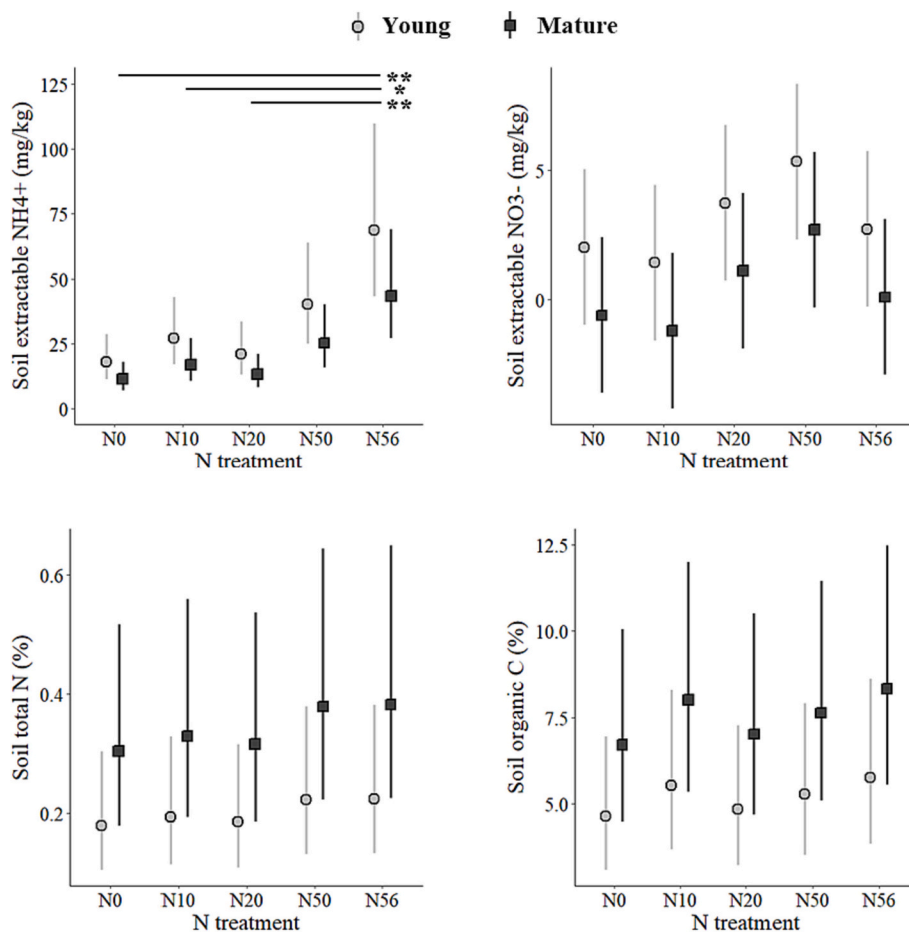


Fig. 1. Mean predicted (95 % confidence intervals) soil nutrients and organic carbon in relation to the effects of heathland ecosystem age (young and mature) and N fertilization treatment (N0, N10, N20, N50, and N56). Significance levels of differences between the levels of each N treatment factor are represented by *** (p-value < 0.001), ** (p-value < 0.01), * (p-value < 0.05), and “none” (p-value > 0.05).

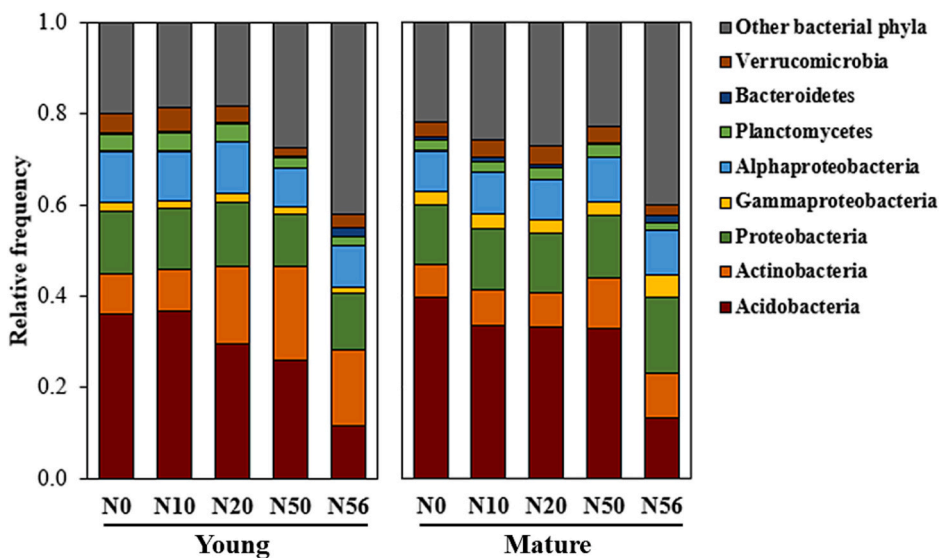


Fig. 2. Relative frequencies of the most representative bacterial taxa in the communities under different N addition treatments. We show bacterial taxa accounting for $\geq 1\%$ of the reads, while those bacterial taxa with a frequency < 1 % are integrated into the “other bacterial phyla” category.

presented higher frequency in soils with high N inputs (from N20 onwards) in young *Calluna* stands (Fig. 2). The other three most frequent phyla corresponded to Verrucomicrobia, Planctomycetes and

Bacteroidetes (Fig. 2). The relative abundances evidenced here agree with those reported for the first soil centimeters in other European *Calluna* heathlands (e.g. Reith et al., 2019; Rineau et al., 2022). Other

phyla that showed high frequency in other forest soils worldwide, such as Chloroflexi and Gemmatimonadetes (Hartmann et al., 2012, 2014; Urbanová et al., 2015; Kaiser et al., 2016), were less represented here. These relative abundances corresponded to intermediate values of the ecological clusters defining bacterial phylotypes with preference for acidic, low productive soils (Delgado-Baquerizo et al., 2018).

Actinobacteria is an extremely diverse phylum, long known to be important contributors to plant biomass decomposition (Waksman, 1931), including lignin metabolism (Ahmad et al., 2010; Berlemont and Martiny, 2013). The most represented orders of this phylum in soil samples of *Calluna* heathlands were Actinomycetales and Solirubrobacterales (Fig. 3), which are the most abundant Actinobacteria in soils (Lewin et al., 2016). The frequency of these two orders, together with Actinobacteria phylum set, strongly increased in N-treated plots, particularly under high N loadings (Fig. 3). This behavior has been previously reported in hardwood forests in western United States under experimental N deposition (Freedman and Zak, 2014; Argiroff et al., 2019). This can be attributable to the copiotrophic (R-selected) strategy of Actinobacteria (Fierer et al., 2007). This strategy is favored under abundant N or labile C in the soil (Eilers et al., 2010; Fierer et al., 2012), which is consistent with the high accumulation of soil extractable N-NH_4^+ under increased N addition evidenced here. For instance, Masse et al. (2017) reported that the availability of readily available N-sources through atmospheric deposition may select copiotrophic taxa such as Actinobacteria (Masse et al., 2017). Additionally, atmospheric N deposition may favor bacterial taxa with high lignin-modifying potential (i.e. lignin degradation to soluble phenolic compounds) such as Actinobacteria (Taylor et al., 2012; Eisenlord et al., 2013; Argiroff et al., 2019).

We found the same behavior in mature *Calluna* stands for the Proteobacteria phylum (Fig. 3), which has been also described as copiotrophic taxa that thrive in conditions of elevated nutrients' availability (Fierer et al., 2007), particularly the three classes with higher sensitivity evidenced here [Alpha-, Beta- (not shown) and Gamma-proteobacteria]. Altogether, our results agree with those of Masse et al. (2017) in boreal forests of western Canada, where they found that the availability of readily available N source from atmospheric deposition, such as extractable N-NH_4^+ , was more important in shaping the composition of microbial communities than the total N or organic C content of soils.

The Acidobacteria phylum is one of the most abundant and ubiquitous in soils of a wide variety of ecosystems worldwide (Eichorst et al., 2018), but the understanding of its role in terrestrial ecosystems remains surprisingly limited at present (Kielak et al., 2016). Among the best represented subdivisions in this study (Fig. 3), GP1 and GP3 have demonstrated a high capacity to use a wide variety of carbohydrates, as well as inorganic and organic N sources, including ammonium, nitrite and nitrate, which constitute advantageous traits in fluctuating, nutrient-poor ecosystems with recalcitrant substrates (Eichorst et al., 2018), such as montane *Calluna* heathlands. Indeed, Acidobacteria is recognized as an oligotrophic (K-selected) bacterial taxa (Fierer et al., 2007; Davis et al., 2011) and the most frequent phylotype in low productive soils (Delgado-Baquerizo et al., 2018). Overall, the frequency of Acidobacteria was strongly reduced with chronic N addition, and, in mature *Calluna* stands, this pattern was also observed in the treatment with high N load (N50) (Fig. 3). Conversely, sequences related to the genus *Granulicella* of Acidobacteria were much more frequent under chronic N addition (Fig. 3), which was consistent with the findings of Turlapati et al. (2015) in mixed hardwood stands in northwestern United States.

Interestingly, the response to N fertilization of the most abundant bacterial taxa at the lower taxonomic level was highly consistent with the response of high-order taxonomic groups (e.g. the phylum-level) in young and mature *Calluna* stands (Fig. 3). Nevertheless, some minor shifts at the lower taxonomic level, such as the high sensitivity of the subclass Acidimicrobiae and lower groups to low N inputs in mature *Calluna* stands, were not always displayed at the higher level due to their low relative abundance as reported by Zeng et al. (2016).

3.3. Alpha diversity

The changes promoted in the soil environment as a consequence of experimental N addition and thus the shifts in the bacterial community composition resulted in a significant response on the community alpha diversity. The richness of bacterial communities was lower in young than in mature *Calluna* stands (Fig. 4; Table 2). However, the N treatment did not affect the rarefied bacterial richness (Table 2).

The effect of N addition on the alpha diversity showed different behavior depending on the age of *Calluna* heathlands (Fig. 4), as shown by the significant interaction between heathland ecosystem age and N fertilization treatment (Table 2). In young *Calluna* stands, the Shannon's diversity decreased progressively (although not significantly) from the control to the N50 treatment, while the plots treated with chronic N additions (N56) presented a significantly higher diversity than in the rest (Table 2; Fig. 4). In contrast, the Shannon's diversity of the bacterial community increased progressively, but not significantly, from the control to the chronic N addition treatment in mature *Calluna* stands (Table 2; Fig. 4). Remarkably, the alpha diversity of the bacterial communities in the control and chronic N addition treatments did not show significant differences between young and mature stands, exhibiting only independent diversity trajectories at intermediate N addition levels (Fig. 4).

These results were consistent with the decrease in the dominance of the most representative bacterial taxa, namely Acidobacteria, under conditions of elevated resource availability (plots with chronic N addition for 10-years). In mature stands of montane *Calluna* heathlands, characterized by high vegetative cover of woody species, Taboada et al. (2018) reported that the annual growth of *Calluna* individuals responded positively to increasing N availability, as did the perennial forbs and graminoids productivity. Moreover, increasing N fertilization rates may promote a higher N and P content in *Calluna* tissues (Calvo-Fernández et al., 2018), translating into cumulative nutrient increases in the litter compartment (Pilkington et al., 2005). These changes, together with the absence of plant diversity losses (Taboada et al., 2018), may procure high inputs of high-quality and diverse litter to the soil (Valencia et al., 2018), which will positively affect the diversity of the bacterial community.

In this context, the observed changes in the dominant bacterial life-history strategies (i.e. oligotroph-copiotroph shifts), may be the result of the increase in readily available N in the organic horizons (Pei et al., 2017) (see Section 3.2 and Fig. 5), as well as from downward translocation arising from the limits to plant N uptake under high resource availability (Pilkington et al., 2005), particularly in mature *Calluna* stands (Barker et al., 2004). Alternatively, the gradual, but no significant increase in organic C availability as a consequence of N addition could have further promoted highly N-demanding copiotrophic taxa at the expense of reduced Acidobacteria dominance (Fierer et al., 2012), and thus the progressive alpha diversity increases reported in mature stands.

In young *Calluna* stands, the gradual decrease in the bacterial alpha diversity with increasing N additions may be associated with the rapid N uptake by plants and increase in the N content of tissues right after N experimental addition in *Calluna* pioneering and building stages (Jones and Power, 2015). In general, young stands have higher investment in green shoots growth and fast-growing tissue than mature ones with higher relatively inert woody material (Britton and Fisher, 2008). The high *Calluna* nutrient demand could induce lower N content available for soil bacterial communities (specifically fast-growing copiotrophic taxa) since *Calluna* plants are assumed to be better competitors for nutrients than soil microorganisms (Harrison et al., 2008). Conversely, cumulative high-N loads in the N56 treatment may exceed vegetation N demands in montane heathlands at early growth vegetation stages (Calvo-Fernández et al., 2018; Taboada et al., 2018) and, therefore, lead to an increase in readily available NH_4^+ , the preferred N source for soil bacterial communities in the organic horizons (Pei et al., 2017). This may explain the significant increase in bacterial alpha diversity in the

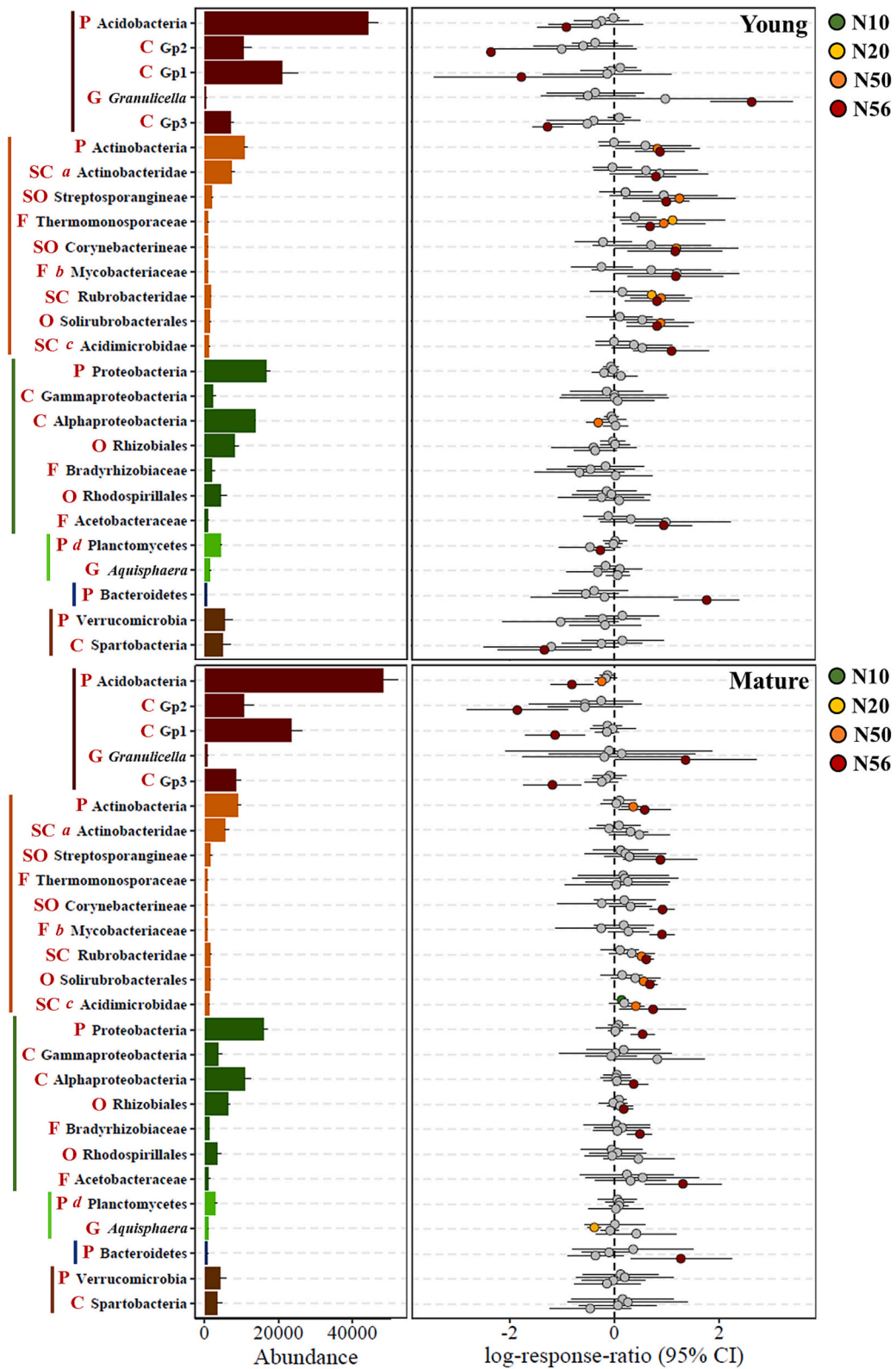


Fig. 3. The abundance (mean and standard deviation) for bacterial taxa accounting for $\geq 1\%$ of the reads in the communities of control samples is listed on the left side. Changes in the relative abundance between N addition treatments as measured by the log-response-ratio (lnRR) with 95 %-confidence interval (CI_{95}) are displayed on the right. Colored points indicate a significant effect of the N dose (N10, N20, N50 and N56) as compared to the control (N0), while gray points denote no significant changes. The bars of taxonomic groups grouped within the same phylum are represented in the same color. Uppercase red letters denote taxonomic groups: phylum (P), class (C), subclass (SC), order (O), suborder (SO), family (F), and genus (G). Lowercase red letters denote: all Actinobacteridae are included in Actinomycetales order (a), all Mycobacteriaceae are included in *Mycobacterium* genus (b), all Acidimicrobidae are included in Acidimicrobiales order (c), and Planctomycetes in Planctomycetes order and Planctomycetaceae family (d).

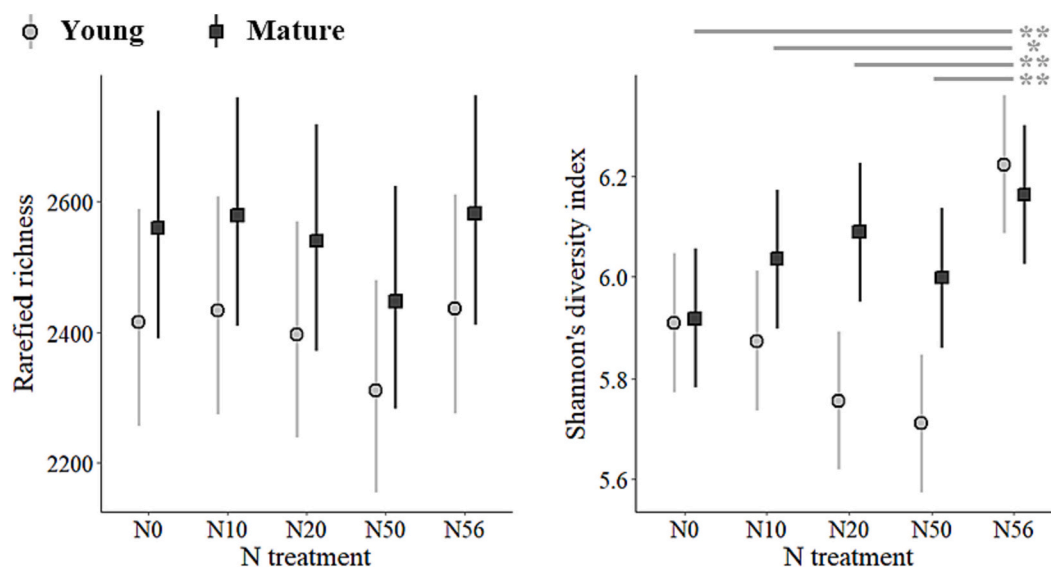


Fig. 4. Mean predicted (95 % confidence intervals) alpha diversity metrics in relation to the effects of heathland ecosystem age (young and mature) and N fertilization treatment (N0, N10, N20, N50, and N56). Significance levels of differences between the levels of each N treatment factor are represented by *** (*p*-value < 0.001), ** (*p*-value < 0.01), * (*p*-value < 0.05), and “ns” (*p*-value > 0.05). There are only significant differences, represented by gray lines, between N treatment levels for young *Calluna* stands.

Table 2

Generalized linear models (GLMs) outputs for the effects of heathland ecosystem age and N fertilization treatment on the alpha diversity metrics of the bacterial communities. The interaction term only was retained in the model when significant. Significant *p*-values are marked in bold. Significance levels are represented by *** (*p*-value < 0.001), ** (*p*-value < 0.01), * (*p*-value < 0.05), and “ns” (*p*-value > 0.05).

Dependent variable	Predictor	Df	F	p-Value (>F)	
Rarefied richness	Age	1	4.049	0.056	ns
	N treatment	4	0.468	0.759	ns
Shannon's diversity index	Age	1	10.935	0.004	**
	N treatment	4	7.011	0.001	**
	N treatment: Age	4	2.977	0.044	*
	Age				

N56 plots, following the same mechanism as in mature *Calluna* stands.

The effect of experimental N addition on the alpha diversity of soil bacterial communities in *Calluna* heathlands has not been previously studied. However, contrasting results have been reported in other forest, shrubland and grassland ecosystems, suggesting that the effects are likely ecosystem- and site-dependent. For example, Zhang et al. (2022) found that the diversity of soil bacterial communities exhibited a slight increase in the long-term (9-years) experimental N addition corresponding to 40 kg N ha⁻¹ yr⁻¹ (the most comparable treatment to N56 in this study) in wetlands of northeastern China. The authors attribute this finding to the increase in soil nutrients at moderate fertilization levels, but in the absence of soil acidification observed with the addition of higher N concentrations (80 kg N ha⁻¹ yr⁻¹), which may produce a change in nutrient availability (Zhalnina et al., 2015; Barrow, 2017) and changes in the fitness of soil bacterial communities (Fierer and Jackson, 2006). The same behavior was also reported in steppe grasslands in China (Liu et al., 2019). Conversely, Fierer et al. (2012) evidenced that bacterial alpha diversity did not correlate with N addition levels (0,34 and 272 kg N ha⁻¹ yr⁻¹) in successional grasslands of northern United States. Zeng et al. (2016) reported only significant decreases in bacterial alpha diversity with the highest N addition treatment (240 kg N ha⁻¹ yr⁻¹) in steppe grasslands of Inner Mongolia, China. In the same region, Wei et al. (2020) found that N addition (100 kg N ha⁻¹ yr⁻¹) significantly hindered the alpha diversity of soil bacterial communities. These

studies attribute alpha diversity shifts to increasing soil acidification and concurrent changes in plant species diversity. However, in *Calluna* heathlands in NW Spain, there were no significant pH changes among treatments, nor in the diversity of plant species (Taboada et al., 2018).

Other studies (e.g. Wang et al., 2021) have shown that bacterial diversity was increased by experimental N addition in forest ecosystems, irrespective of the significant decrease in soil pH due to high N depositions (50–150 kg N ha⁻¹ yr⁻¹), which give light to the ecosystem- and site-dependent nature of N fertilization effects on soil bacterial communities. Therefore, further research is needed to unravel the ecological mechanisms behind the shifts in soil bacterial communities in montane *Calluna* heathlands observed here.

3.4. Beta diversity

PCoA of beta diversity indices (Chao, Bray-Curtis, un-weighted UniFrac and weighted UniFrac) identified changes in the community composition between ecosystem age and N treatment levels (Fig. 6). The first PCoA component splits communities treated with long-term N addition (N56) from the rest of the treated (N10, N20 and N50) and control (N0) communities, which are clustered together, indicating similar bacterial community composition (Fig. 6). The second PCoA component for all beta diversity indices was clearly related to the ecosystem age (Fig. 6). When comparing Chao (qualitative) and Bray-Curtis (quantitative) indices, the pattern shown by the communities is mostly identical. These results confirmed that N addition, specifically chronic N addition treatment in the long-term, had relevant effects on the overall composition of soil bacterial communities. In the UniFrac metrics, besides changes in abundance, the phylogenetic distance between OTUs not shared between communities is also considered. The increase in variance explained by the first axis of weighted UniFrac with respect to Bray Curtis indicates that changes in abundance occur in OTUs that are not always close in the phylogenetic tree (Sáenz de Miera et al., 2020) and thus would belong to different taxonomic groups fulfilling distinct functions and showing distinct environmental adaptations (Lozupone and Knight, 2005). These changes affected communities in soils treated with long-term chronic N addition.

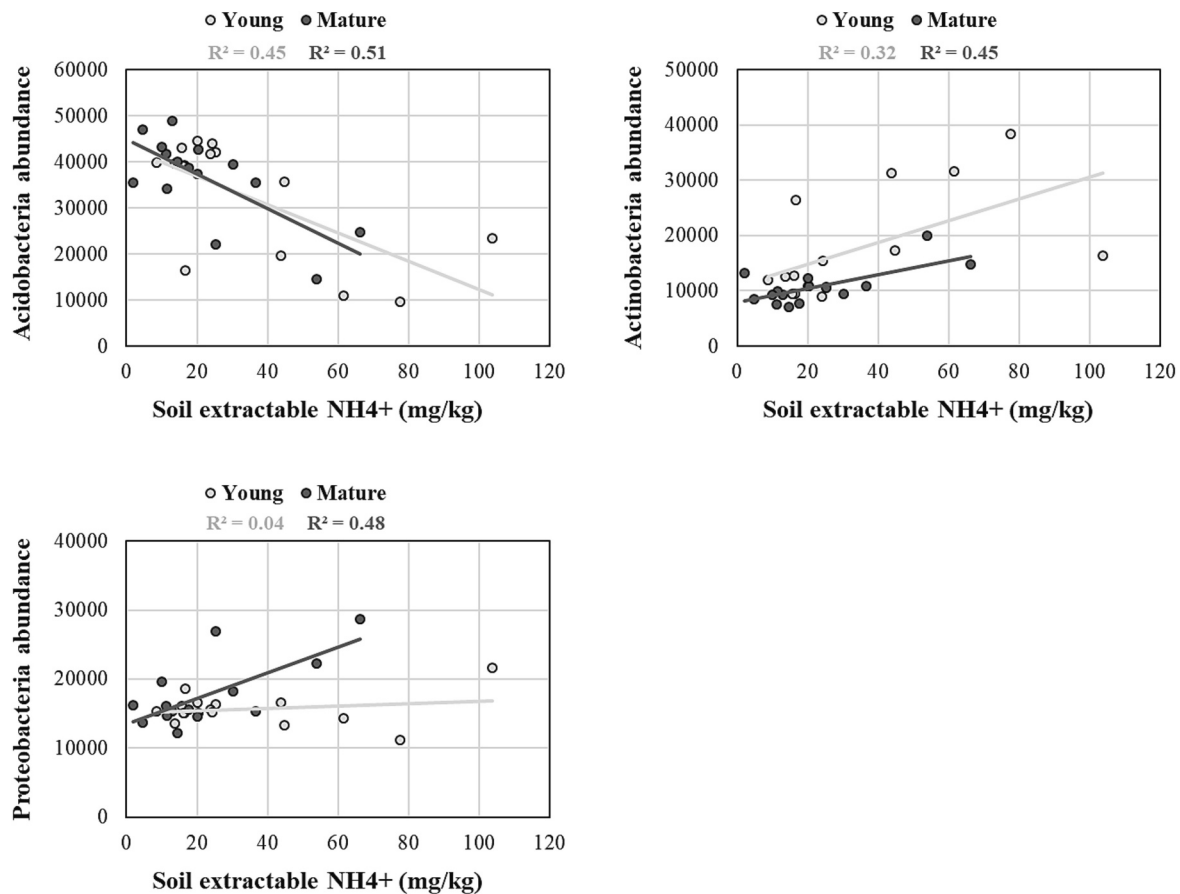


Fig. 5. Relationships between soil extractable N-NH_4^+ , the individual soil property significantly affected by N fertilization treatment, and the bacterial phyla showing the greatest changes in abundance among treatments for young and mature stands.

4. Conclusions

This study provided novel evidence on the behavior of soil bacterial communities in response to experimental N addition, improving the understanding of the role of atmospheric nitrogen deposition with respect to the functioning of montane *Calluna* heathlands in the context of global change. We found differential N addition effects on taxonomic groups of the soil bacterial community with markedly different functions within the ecosystem. Indeed, the increase in N availability modified the bacterial community composition with an increase in the copiotrophic taxa such as Actinobacteria, with high lignin-modifying potential. The alpha and beta diversity of bacterial communities showed different resistance to nitrogen addition depending on the maturity of the *Calluna* ecosystem. The bacterial communities of mature *Calluna* stands were resistant to nitrogen addition (hypothesis two), whereas the communities of young stands showed shifts in their diversity once a certain intensity of the disturbance is exceeded (hypothesis three), in particular under long-term chronic nitrogen addition. These shifts were mainly due to a decrease in the dominance of Acidobacteria, the most representative phylum in montane *Calluna* ecosystems, in favor of copiotrophic taxa such as Actinobacteria or Proteobacteria, favored under increased soil nutrients' availability, particularly ammonium. Future research is needed to unravel if the feedbacks evidenced here are consistent at longer term or under higher experimental nitrogen addition loads. In addition, it is necessary to discover what specific functions of the soil bacterial community may be sensitive to increased nitrogen depositions, which may have important implications for the understanding of montane *Calluna* ecosystems' stability.

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CRedit authorship contribution statement

José Manuel Fernández-Guisuraga: Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Gemma Ansoła:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Rayo Pinto:** Writing – review & editing, Investigation, Formal analysis. **Elena Marcos:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Leonor Calvo:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization. **Luis E. Sáenz de Miera:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis.

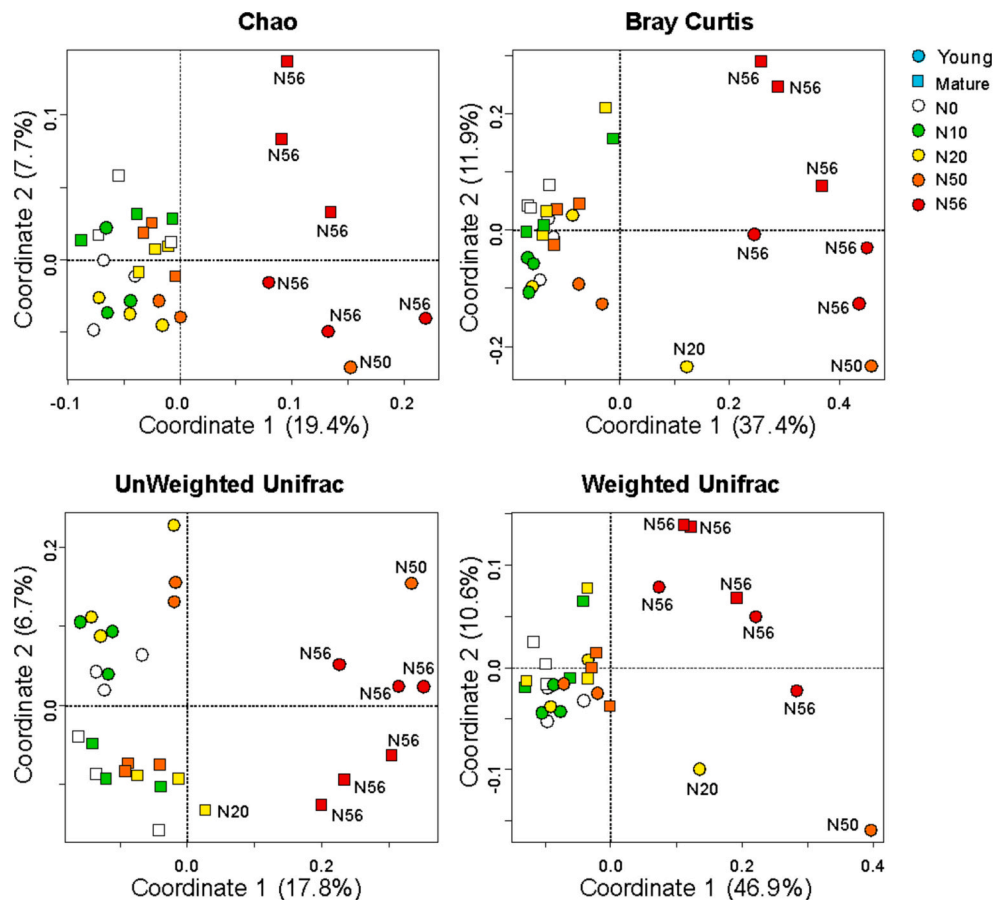


Fig. 6. Ordination of the bacterial communities along ecosystem age (young and mature) and N treatment levels (N0, N10, N20, N50, N56) using principal coordinates analysis (PCoA) of beta diversity indices.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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