





# The added value of including key microbial traits to determine nitrogen-related ecosystem services in managed grasslands

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## Abstract

1. Despite playing central roles in nutrient cycles and plant growth, soil microbes are generally neglected in the study of ecosystem services (ES), due to difficulties to assess their diversity and functioning. However, to overcome these hurdles, new conceptual approaches and modern tools now provide a means to assess the role of micro-organisms in the evaluation of ES.
2. In managed grasslands, soil microbes are central in providing nitrogen (N)-related ES such as maintenance of soil fertility and retention of mineral forms of N. Here, we applied state-of-the-art techniques in microbial ecology and plant functional ecology to uncover the intrinsic link between N-related bacterial functional groups, important plant functional traits, environmental factors and three proxies of maintenance of soil fertility and potential for N-leaching across managed grasslands in three regions of Europe.
3. By constructing well-defined structural equation modelling, we showed that including key microbial traits improve on average more than >50% of the total variances of ES proxies, that is, ammonium ( $\text{NH}_4^+$ ) or nitrate ( $\text{NO}_3^-$ ) leaching, and soil organic matter content. Geographic differences arose when considering the direct relationships of these ES proxies with specific microbial traits: nitrate leaching was positively correlated to the maximum rate of nitrification, except in the Austrian site and potentially leached  $\text{NH}_4^+$ -N was negatively correlated to the fungi/bacteria ratio, with the exception of the French site.
4. *Synthesis and applications.* The integration of soil microbial functional traits in the assessment of nitrogen-related grassland ecosystem services has direct contributions for understanding sustainable management of grassland ecosystems. The fundamental aspects of this study suggest that integrating a soil microbial component in grassland management may enhance sustainability of such grass-based agroecosystems.

## KEYWORDS

above- and below-ground interactions, agroecology, ecosystem services, functional traits, grass-based agroecosystems, N-cycle, nitrogen retention, soil fertility, subalpine grassland

## 1 | INTRODUCTION

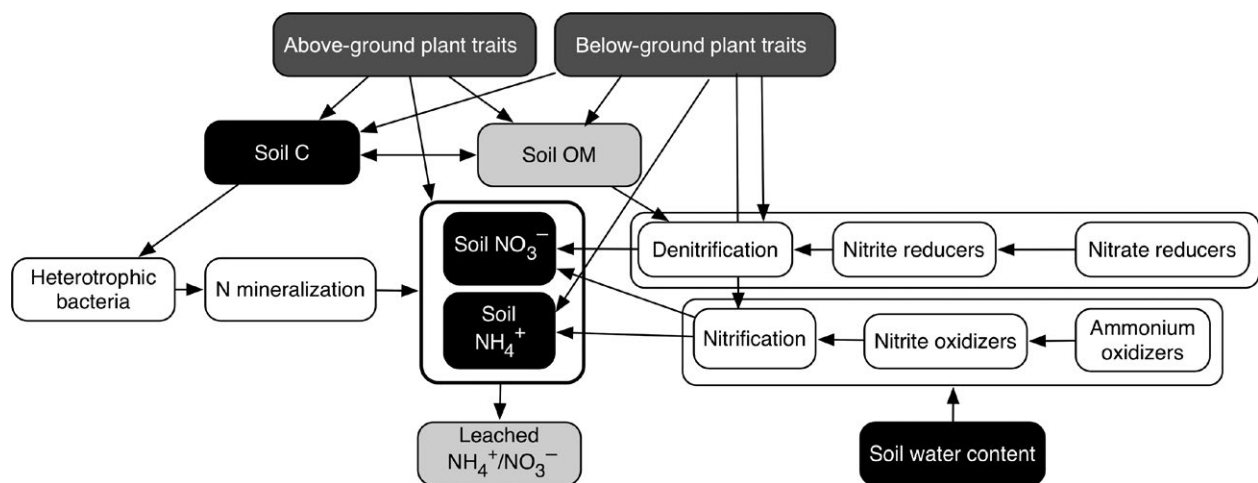
The concept of ecosystem services (ES) emphasizes the intrinsic values of nature as constituents of human well-being (Leemans & de Groot 2003). Because agroecosystems—including grasslands and croplands—account for one of the largest terrestrial biomes (Foley et al., 2005), and because food and fiber production are often associated with negative impacts on other critical services through radical environmental shifts in nitrogen and phosphorous cycles (Garbach, Milder, Montenegro, Karp, & DeClerck, 2014), evaluating how management of agroecosystems impact the provisioning of ES has become a high priority for researchers. However, since its emergence over the past three decades, assessments of ES have essentially focused on large living organisms or geochemical entities and neglected the importance of microbes in several aspects (Garbach et al., 2014; Zhang, Ricketts, Kremen, Carney, & Swinton, 2007).

Except for a recent study at a global scale (Delgado-Baquerizo, Maestre, et al., 2016), microbes are usually considered within the biotic part of the abiotic-biotic soil black box that contribute to supporting services, such as nutrient cycling, primary production, or food and fiber production, regardless of their specific attributes such as metabolic optima or growth dynamics (Hayatsu, Tago, & Saito, 2008; Saggar et al., 2013). Further, when assessing biodiversity and its link to ES, most researchers have disregarded this important attribute of the biota, despite its potential to strongly impact ecosystem processes (Van der Heijden, Bardgett, & van Straalen, 2008). Nevertheless, the importance of microbes in ES assessment is not limited to supporting services (e.g. dairy or alcoholic beverages production) and numerous examples suggest a need to widen our vision of their role in ES delivery, including the limitation of antibiotic use in cattle production to sustained water quality regulation in wetland ecosystems (Peralta, Stuart, Kent, & Lenno, 2014), or monitoring the effects of plant community changes in agroecosystems that affect soil organic matter (SOM) dynamics and feedback systems (Lemaire, Wilkins, & Hodgson, 2005).

The complexities in characterizing microbial functioning and diversity paired with ongoing technical limitations (Van der Heijden et al., 2008) may explain this lack of inclusion in ES delivery. First, the vast majority of microbial diversity has proved resistant to cultivation (Pace, 1997), which had strong consequences on our understanding of the dynamics of this part of the biota. Molecular biology, as well as the advances of next-generation sequencing, has revolutionized our perspectives and it is now conceivable to precisely describe the various parts of microbial diversity (Doney, Abbott, Cullen, Karl, & Rothstein, 2004). Second, the level of functional redundancy is often assumed to be high for microbes (Nannipieri et al., 2003) because of their ability for dispersion and their immense estimated diversity (Curtis, Sloan, & Scannell, 2002). Nevertheless, such redundancy may also vary depending on the environments since microbial taxa carrying out the same functions may perform those functions at different rates (Strickland, Lauber, Fierer, & Bradford, 2009) and alter the biodiversity-ecosystem functioning relationship (Delgado-Baquerizo, Giaramida, et al., 2016). Third, the capacity of most micro-organisms to transfer genes horizontally has also hindered our capacity to apply the concept of species. Assigning functions to specific cells or populations is, therefore, particularly complex, and quantifying the relative contribution of specific microbes to distinct ES is problematic. Although phylogenetically related microbes may share certain microbial functional traits (Martiny, Jones, Lennon, & Martiny, 2015), a potential solution to this intricacy is to measure microbial functional traits in a similar manner as aggregated traits measured by plant ecologists (Allison, 2012; Lavorel, McIntyre, Landsberg, & Forbes, 1997) in order to define functional groups that may contribute to various ES.

### 1.1 | Rationale of key microbial traits involved in two soil ES

We propose here a conceptual framework that integrate solid knowledge on the relationships between above-ground and



**FIGURE 1** *A priori* model for three proxies of the maintenance of soil fertility and water quality (light grey boxes) and their relationships with microbial (white boxes) and plant (dark grey boxes) functional traits and soil characteristics (black boxes). Arrows indicate either positive or negative relationships, depending on the considered variables

below-ground plant traits, soil biogeochemistry and microbial traits measured at the community level to explain the variance in three proxies for the maintenance of soil fertility and the retention of mineral forms of N (Figure 1). First, we estimated the relative contribution of plant and microbial traits in relation to soil fertility by focusing on the variation in SOM (Dominati, 2013; Robinson et al., 2013). Hence, high specific leaf area (SLA; Grime, 1977), leaf N content (Orwin et al., 2010) or root N concentration (Roumet, Urcelay, & Diaz, 2006), which are representative of exploitative plant species, are usually associated with grasslands harbouring high concentration of SOM, total soil C and N (Lavorel & Garnier, 2002). Second, we measured potentially leached ammonium ( $\text{NH}_4^+$ -N) and nitrate ( $\text{NO}_3^-$ -N) which represent good proxies for soil mineral N-retention. While nitrification and denitrification processes directly influence N-leaching, there are also growing evidences that specific root functional traits directly affect these microbial soil processes (Cantarel et al., 2015; Legay et al., 2016; Moreau et al., 2015), their relative abundances (Thion et al., 2016) or through the release of labile C sources (Kuzakov, 2010). Plant roots, therefore, directly drive the amount of N in soil but also indirectly affect soil N-retention through their regulating activities on microbial processes (Hayatsu et al., 2008). As an anaerobic-facultative process, denitrification is performed by microbial heterotrophs that are sensitive to pH, nitrate and organic matter levels and soil water content (Koskinen & Keeney, 1982; Simek, Jisova, & Hopkins, 2002; Zumft & Korner, 1997). Nitrification is a less common autotrophic process that is driven mostly by soil ammonium concentrations and soil water content (Patra et al., 2007; Prosser, 1989). These two important processes of the soil N-cycle are obviously driven by the relative abundances of functional groups performing key enzymatic steps, that is, nitrite and nitrate reducers for denitrification and ammonium and nitrite oxidizers for nitrification. Ultimately, the variations of potential leaching of ammonium and nitrate, as well as SOM content variance, are related to the different biotic and abiotic drivers described above.

Here, to estimate the relative contribution of microbes to nitrogen-related ES in mountain managed grasslands, we applied this conceptual framework to eight different sampling fields across Europe. This broad perspective documents such dynamics in different grass-based agroecosystem under common climatic conditions of the Northern Hemisphere. We tested the hypothesis that integrating the measures of microbial traits would significantly improve the amount of variance explained for the ES proxies considered. We modeled how these key microbial traits (i.e. (de)nitrifying enzymatic activities, gene abundances of key functional groups) interact with important soil biogeochemical characteristics and essential plant functional traits. We, hence, complement the approach presented by Grigulis et al. (2013) that aimed at measuring the relative importance of the environmental processes controlled by microbes in three mountain grassland systems across Europe. We discuss our results in the light of biomass production, and its links with N-related and microbial driven ES, to provide prescriptions for grass-based agroecosystems and grassland management.

## 2 | MATERIALS AND METHODS

### 2.1 | Overview of study sites and sampling

We sampled soil and vegetation in three replicates from two or three 1,000 m<sup>2</sup> wide individual mountain grasslands fields at peak plant biomass in summer 2010, along a range of climatic, geomorphologic, and land-use conditions at three long-term research sites, as described in details in Grigulis et al. (2013). These sites were as follows: Austria Tyrol ("Stubai" henceforth); two grasslands (the first abandoned and the second cut once a year, grazed in late summer, and manured every 2–3 years); northern England ("Yorkshire Dales" henceforth); three grasslands (the first cut once or twice a year, high-intensively grazed, manured once a year, the second, cut annually, medium-intensively grazed, manured every 2 years, and the third cut annually, low-intensively grazed, without fertilizer), and in the French Alps ("Lautaret" henceforth); three grasslands (the first fertilized and mown, the second unmown and grazed in spring and autumn and the third unmown and summer grazed—dominated by large perennial grasses).

Within each field, we sampled soil and vegetation in four 50 × 50 cm quadrats. Ninety-six experimental units were, therefore, considered. Six soil cores (4.5 cm Ø, 0–10 cm deep) were sampled from each quadrat and sieved at 4 mm; four cores were subsequently pooled for soil and microbial analyses, a single core was taken for root trait analyses (see details below), and one final core was intended for measurement of bulk density, soil water availability, and potential for ammonium and nitrate leaching.

### 2.2 | Plant, microbial and soil descriptions

Total above-ground biomass, standing litter and vegetation composition were estimated using the BOTANAL method (Tothill, Hargreaves, Jones, & McDonald, 1992) and plant functional traits, that is, total above-ground biomass, standing litter and vegetation composition, vegetative height (VH), specific leaf area (SLA), leaf dry matter content (LDMC), leaf C and N concentrations (LCC and LNC) were measured following Pérez-Harguindeguy et al. (2013) for each of the species that collectively made up 80% of the cumulated biomass. Community-weighted means (CWM) were calculated for each plant trait. CWM roots traits (i.e. total root length, average root diameter, root dry matter content [RDMC], C and N contents [RNC, RCC] and specific root length [SRL]) were measured on a dedicated soil core in each quadrat. Root morphology (total root length and average root diameter) was assessed using digital scanning and WINRHIZO software (Regent Instruments Inc., Quebec city, QC, Canada). Since this study aimed to highlight the relative contribution of microbial properties to the environmental processes, the measured root functional traits were assumed as good proxies of nutrient acquisition and different classes of root orders and diameter were not considered. Indeed, root biomass and SRL together reflect a plant's ability to prospect soil and take up nutrients (Craine, Wedin, Chapin, & Reich, 2003; Legay, Personeni,

Slezack-Deschaumes, Piutti, & Cliquet, 2014) and root N content is correlated with root branching, which is also a well-established proxy of nutrient uptake potential (Liese, Alings, & Meier, 2017).

Soil and microbial analyses were performed on subsamples from the pool of four soil cores. Freshly sieved subsample was frozen at  $-20^{\circ}\text{C}$  for subsequent DNA extraction and phospholipid analyses. Remaining fresh soil was taken back to the lab in cool boxes (c.  $4^{\circ}\text{C}$ ) for subsequent measures of microbial biomass and potential (de)nitrification enzymatic activities, soil moisture, SOM content, pH, total N and total C. Maximum rate ( $V_{\text{max}}$ ) and half substrate saturation ( $K_m$ ) of potential nitrification enzyme activity (NEA) were determined according to Koper, Stark, Habteselassie, and Norton (2010) and following Dassonville, Guillaumaud, Piola, Meerts, and Poly (2011). Briefly, 3 g dried wet from each composite fresh soil sample from each quadrat was incubated under aerobic conditions (180 rpm,  $28^{\circ}\text{C}$ , 10 h) in a solution of  $(\text{NH}_4)_2\text{SO}_4$  (0, 0.5, 1, 2, 5 and 15 mg N/L). Rates of  $\text{NO}_2^-$  and  $\text{NO}_3^-$  production were measured after 2, 4, 8 and 10 hr by ionic chromatography (DX120; Dionex, Salt Lake City, UT, USA). As detailed in Cantarel et al. (2015), maximal nitrification rate ( $V_{\text{max}}$ ) and  $\text{NH}_4\text{-N}$  affinity ( $1/k_m$ ) were assessed by plotting nitrification rates along the gradient of  $\text{NH}_4\text{-N}$  concentrations (Lineweaver & Burk 1934). Potential denitrification enzyme activity (DEA) was measured according to Attard et al. (2011). Briefly, c. 10 g dry weight (dw) soil was placed at  $28^{\circ}\text{C}$  under anaerobic conditions using 90:10  $\text{He}:\text{C}_2\text{H}_2$  mixture inhibiting  $\text{N}_2\text{O}$ -reductase activity. Each flask was supplemented with c. 3 ml  $\text{KNO}_3$  (50 mg  $\text{N-NO}_3^- \text{g}^{-1} \text{dw}$ ), glucose (0.5 mg C/g dw) and sodium glutamate (0.5 mg C/g dw), completed with distilled water to reach the water-holding capacity.  $\text{N}_2\text{O}$  was measured at 2, 3, 4, 5 and 6 hr using a gas chromatograph (microGC R3000; SRA Instruments, Marcy l'Etoile, France). Although performing DEA without  $\text{C}_2\text{H}_2$  could be very interesting to evaluate whether incomplete denitrification to  $\text{N}_2\text{O}$  was important to the total denitrified  $\text{N-NO}_3^-$  and when considering contribution of denitrifiers to climate mitigation ( $\text{N}_2\text{O}$  is a greenhouse gas with an effect c. 300 times higher than  $\text{CO}_2$ ), focus was set on nitrate leaching only. Soil DNA was extracted from 0.5 g of fresh soil using the FastDNA SPIN Kit for soil (MP Biomedicals, Irvine, CA, USA) and a Precellys 24 Instrument (Bertin Technologies, Montigny-le Bretonneux, France). Quantity and quality of extracted DNA were tested by spectrophotometry (Nanodrop; PeqLab, Erlangen, Germany). Abundances of ammonium-oxidizing bacteria (AOB) and archaea (AOA) and nitrite oxidizers (*Nitrospira* spp.) were quantified based on the gene copy numbers present per g of dry soil of the ammonium monooxygenase gene *amoA* (for both bacteria and archaea) and 16S rRNA gene specific to *Nitrospira* spp. and *nrxA* gene *Nitrobacter* spp., respectively. Abundances of denitrifying bacteria were measured based on the abundance of the nitrite reductase genes *nirS* and *nirK*. Duplicate quantitative real-time PCR (qPCR) were carried out using SYBR green as the fluorescent dye on a Lightcycler 480 (Roche Diagnostics, Meylan, France). Efficiencies of all performed qPCRs were in the range 85%–99%.  $R^2$  values were always above 0.95. Possible inhibition of PCR was tested in advance and appropriate dilutions were chosen (data not shown).

After 7 days of anaerobic incubation in the dark, potential N mineralization rates (PMN) were determined by subtracting  $\text{NH}_4^+\text{-N}$

contents mineralized and accumulated from initial measures (Waring & Bremner, 1964; Wienhold, 2007). Soil water (SWC) and organic matter content (SOM%) were obtained on subsamples of fresh soil by loss on ignition technique; that is, after a drying period of 1 week at  $70^{\circ}\text{C}$ , to determine SWC, the subsamples followed 4 hr at  $550^{\circ}\text{C}$  to determine SOM%. Potentially leached  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_3^-\text{-N}$  were calculated from percolates of the fifth soil core/quadrat leached with a given volume of distilled water (De Vries, van Groenigen, Hoffland, & Bloem, 2011).

Soil texture analyses were done at the Laboratoire d'Analyses des Sols of the Institut National de la Recherche Agronomique in Arras, France, following a modern version (NF X 31-107, ISO 9001-certified) of the so-called pipette method of Robinson (1923). Briefly, soil was sieved at 2 mm and subsequently treated with  $\text{H}_2\text{O}_2$  to remove organic matter. Then, coarse sands were sieved at 0.2 mm. The remaining soil was dispersed using  $[(\text{NaPO}_3)_6 + \text{Na}_2\text{CO}_3]$  and micro-waves. Weighing after evaporation and drying of the fractions sampled with the pipette enabled the determination of four other granulometric grain sizes (fine sands 0.05–0.2 mm, coarse loam 20–50  $\mu\text{m}$ , fine loam 2–20  $\mu\text{m}$ , clay <2  $\mu\text{m}$ ). Soil subsamples were air dried and ground to measure total soil C and N with a FlashEA 1112 elemental analyser (Fisher Scientific Inc., Waltham, MA, USA) and soil pH was measured in a 1:5 (soil/distilled water) solution. Bulk density and soil porosity were obtained by measuring the dry mass of a fixed-volume (205  $\text{cm}^3$ ) soil core. Prior to drying, 100-ml distilled water was added to saturate each soil core and allowed us to calculate water-holding capacity and water-filled pore space. Phospholipid fatty acid (PLFA) analysis was performed to assess fungal and bacterial biomass ratio (Bardgett, Hobbs, & Frøstegård, 1996), which informs on the relative biomasses of fungi and bacteria from the same soil sample (Van der Heijden et al., 2008). Chloroform-fumigation extraction technique was used to estimate microbial biomass C and N (Vance, Brookes, & Jenkinson, 1987).

## 2.3 | Statistical analyses and structural equation modelling

All variables were tested for normality, and log or square root transformations applied, as required, prior to analysis. Variable selections followed a hypothesis-driven process, using linear mixed models with restricted maximum likelihood (REML) estimates according to Grigulis et al. (2013). This approach allows the specification of covariance structure induced by grouping dependent variable within each site and provides good estimations of parameter effects and variance components for both random (site) and fixed (soil properties, plant and microbial traits) response variables in the model of proxies of ES. Analyses were carried out using the average information (AI) algorithm to estimate variance parameters using the software package JMP 12.0 (SAS Institute, Cary, NC, USA). Then, as described by Grigulis et al. (2013), each response variable was added to the model in a stepwise manner, with those variables significant in the presence of previously fitted variables being retained in the model, and variables no longer significant in the presence of other variables, due to co-linearity, being removed from the model. Structural equation modelling was then used to explore mechanistically based on *a priori* hypothetical models of causal

effects between the retained variables from the REML describing microbial and plant functional traits, soil properties and three proxies of the maintenance of soil fertility and water purification (Shipley, 2000). This robust statistical method tests how experimental data fit a hypothesized causal structure that is well suited for investigating interactions between multiple traits and ecosystem functioning based on prior knowledge (Garcia-Palacios, Maestre, & Milla, 2013). Using the Amos18 (Amos Development Corporation, Crawfordville, FL, USA), *a priori* models were thus constructed based on our hypotheses and theoretical knowledge of plant-microbe controls on N uptake and leaching and soil-microbial interactions in typical grasslands (Figure 1).

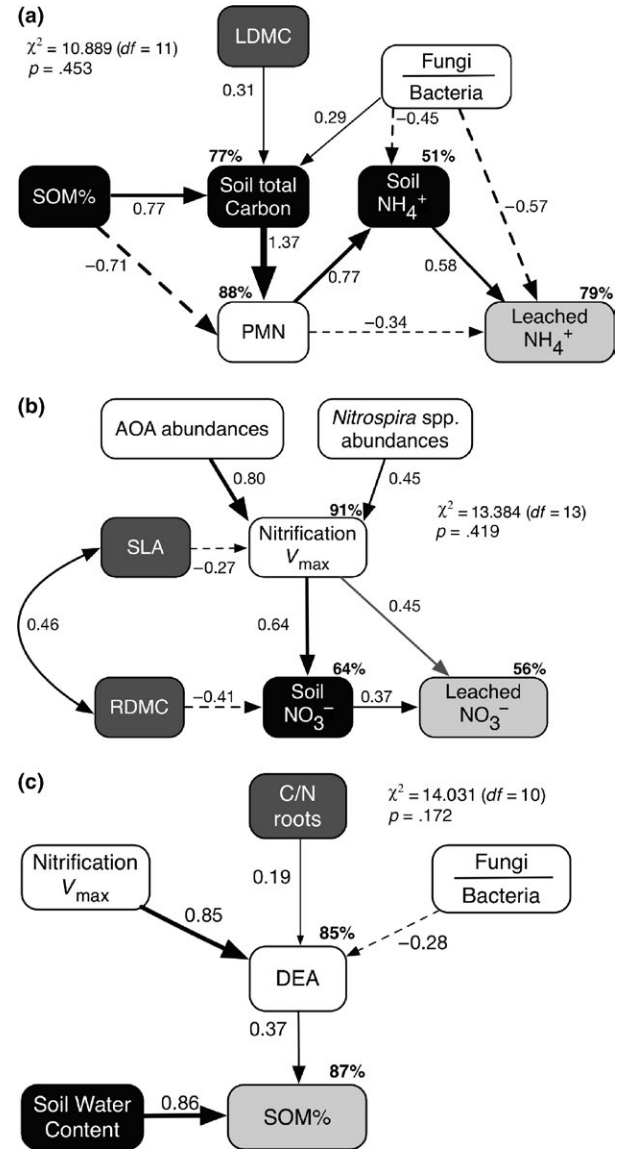
To explore the added value of including microbial traits to ES provision, we first fitted models including only soil properties and above-ground and below-ground plant functional traits, after which we fitted models including all soil properties, plant and microbial traits. We recursively removed variables that show non-significant relationships and tested the effect of these removals on Akaike information criterion and model fit using a likelihood ratio test as recommended by Shipley (2013). Adequate model fits were tested and retained when  $\chi^2$  test was non-significant ( $p > .05$ ). To assess robustness of our structural equation modelling (SEMs), two-third of the dataset was randomly sampled 25 times (with replacement), and the SEM was performed again to confirm that the  $\chi^2$  test remained non-significant.

Site specificity was explored by searching for linear correlations between the considered proxies (i.e. SOM% and potential leached  $\text{NH}_4^+\text{-N}$  and  $\text{NO}_3^-\text{-N}$ , transformed to follow normal distribution) and the direct variables related to microbial traits.

### 3 | RESULTS

#### 3.1 | SEMs for three proxies of two ecosystem services

We built three specific structural equation models (SEMs) to reveal how soil properties and plant and microbial functional traits directly or indirectly influence the potential leaching of  $\text{NH}_4^+\text{-N}$  (Figure 2a),  $\text{NO}_3^-\text{-N}$  (Figure 2b) and SOM content (Figure 2c). All tested variables are presented in Table S1 and the retained variables in Table 1. The respective SEM fitted the data well (see below for each respective fit) and showed that including microbial traits strongly improved the amount of variance explained for the different ES proxies considered. The best SEM explained the variance of potential for  $\text{NH}_4^+\text{-N}$ -leaching ( $\chi^2 = 10.889$ ,  $df = 11$ ,  $p = .453$ ) at 79% (Figure 2a). When excluding the microbial specific traits, the best model would only explain 60% of this variance ( $\chi^2 = 7.44$ ,  $df = 5$ ,  $p = .19$ ). The correlation matrix of SEM variables is detailed in Table S2. As expected plant leaf dry matter content, total SOM% and the fungi/bacteria (F/B) ratio directly affected the amount of total C in soil, which in turn controlled the amount of soil ammonium concentrations. These three components explained 77% of the variance of total soil C. Potential  $\text{NH}_4^+\text{-N}$  leaching was directly and positively linked to Soil  $\text{NH}_4^+\text{-N}$  concentrations and negatively to the fungi/bacteria PLFA ratio and potential mineralization of N (PMN). The F/B PLFA ratio was negatively related to soil  $\text{NH}_4^+\text{-N}$  concentrations. Eighty-eight percent of PMN variance was explained through its



**FIGURE 2** Structural equation modelling (SEM) for soil potential for  $\text{NH}_4^+\text{-N}$ -leaching (a), soil potential for  $\text{NO}_3^-\text{-N}$ -leaching (b) and soil organic matter content (c). Soil abiotic characteristics, microbial traits, plant traits and proxies for ecosystem services are represented in black, white, dark grey and light grey rectangles, respectively. Percentages of explained variances of node variables are indicated in the top-right corner. Plain and dash arrows indicate positive and negative relationships, respectively. Figures above arrows and arrows weight indicate the strength of the relationships. Chi-squared statistics, degree of freedom ( $df$ ) and  $p$ -value of the models are indicated for each SEM.  $p > .05$  indicate a significant model

correlations with total soil C contents and SOM%. Moreover, because PMN also positively influenced soil  $\text{NH}_4^+\text{-N}$  concentrations, there was a double effect of PMN on potential  $\text{NH}_4^+\text{-N}$ -leaching, with a direct negative influence and an indirect influence through soil  $\text{NH}_4^+\text{-N}$ .

Our SEM explained at best 56% of variance in potential for  $\text{NO}_3^-\text{-N}$ -leaching (Figure 2b) ( $\chi^2 = 13.384$ ,  $df = 13$ ,  $p = .419$ ). When excluding the microbial specific traits, the best model only explained 47% ( $\chi^2 = 0.192$ ,  $df = 3$ ,  $p = .979$ ) of this variance, indicating that microbial traits were important to our understanding of soil N-leaching.



Variables in SEM	Abbreviations	Units
<i>Microbial functional traits</i>		
Fungal to bacterial PLFA ratio	F/B ratio	
Abundances of ammonia-oxidizing Archaea	AOA	nb of amoA gene copies g <sup>-1</sup> dry soil
Abundances of nitrite oxidizers ( <i>Nitrospira</i> spp.)	<i>Nitrospira</i> spp. abundances	nb of NS 16S gene copies g <sup>-1</sup> dry soil
Potential maximal rate of nitrification enzyme activity	V <sub>max</sub>	μg N-NH <sub>4</sub> <sup>+</sup> g <sup>-1</sup> dry soil h <sup>-1</sup>
Potential denitrification enzyme activity	DEA	μg N-N <sub>2</sub> O g <sup>-1</sup> dry soil h <sup>-1</sup>
Potential mineralization of nitrogen	PMN	μg N-NH <sub>4</sub> <sup>+</sup> g <sup>-1</sup> dry soil d <sup>-1</sup>
<i>Plant functional traits</i>		
Leaf dry matter content	LDMC	mg dry mass g <sup>-1</sup> leaf fresh mass
Specific leaf area	SLA	m <sup>2</sup> of leaf kg <sup>-1</sup> leaf dry mass
Root dry matter content	RDMC	mg dry mass g <sup>-1</sup> root fresh mass
Root carbon to nitrogen ratio	C/N root	
<i>Soil properties</i>		
Soil ammonium content	Soil NH <sub>4</sub> <sup>+</sup>	μg NH <sub>4</sub> <sup>+</sup> -N g <sup>-1</sup> dry soil
Soil nitrate content	Soil NO <sub>3</sub> <sup>-</sup>	μg NO <sub>3</sub> <sup>-</sup> -N g <sup>-1</sup> dry soil
Soil organic matter content	SOM%	%
Soil water content	SWC	%
Total soil carbon content	Soil total C	μg C g <sup>-1</sup> dry soil
Potentially leached soil ammonium	Leached NH <sub>4</sub> <sup>+</sup>	kg <sup>-1</sup> ha <sup>-1</sup>
Potentially leached soil nitrate	Leached NO <sub>3</sub> <sup>-</sup>	kg <sup>-1</sup> ha <sup>-1</sup>

**TABLE 1** Microbial and plant functional traits and soil properties and processes retained for structural equation modelling with their abbreviations and units

Two key plant functional traits, that is, SLA and RDMC, negatively impacted nitrification maximum rate and soil NO<sub>3</sub><sup>-</sup>-N concentration, respectively. The abundances of ammonia-oxidizing Archaea (AOA) and *Nitrospira* spp. strongly impacted nitrification maximum rate, explaining 91% of variance in this measure. This variable directly impacted soil nitrate content (64% variance explained) and potential leaching of nitrate.

Variance in SOM% was largely (87%) explained by the direct impacts of both soil water content and DEA (Figure 2c;  $\chi^2 = 14.031$ ,  $df = 10$ ,  $p = .172$ ). Excluding microbial trait measures from this model resulted in a single link between soil water content and SOM%, which cannot be represented as SEM. DEA was positively linked to the maximum rate of nitrification and to root C/N. Simultaneously, the F/B ratio negatively impacted DEA, which variance was explained at 85% by this combination of plant and microbial traits.

Robustness of each SEM did not vary significantly when performing the  $\chi^2$  test on 25 random subsamples of the datasets (Figure S1). Eighty-eight percent, 76% and 84% of the simulations showed a  $p > .05$  in the NO<sub>3</sub><sup>-</sup>-N-leaching, NH<sub>4</sub><sup>+</sup>-N-leaching and %SOM, respectively.

### 3.2 | Geographic variations

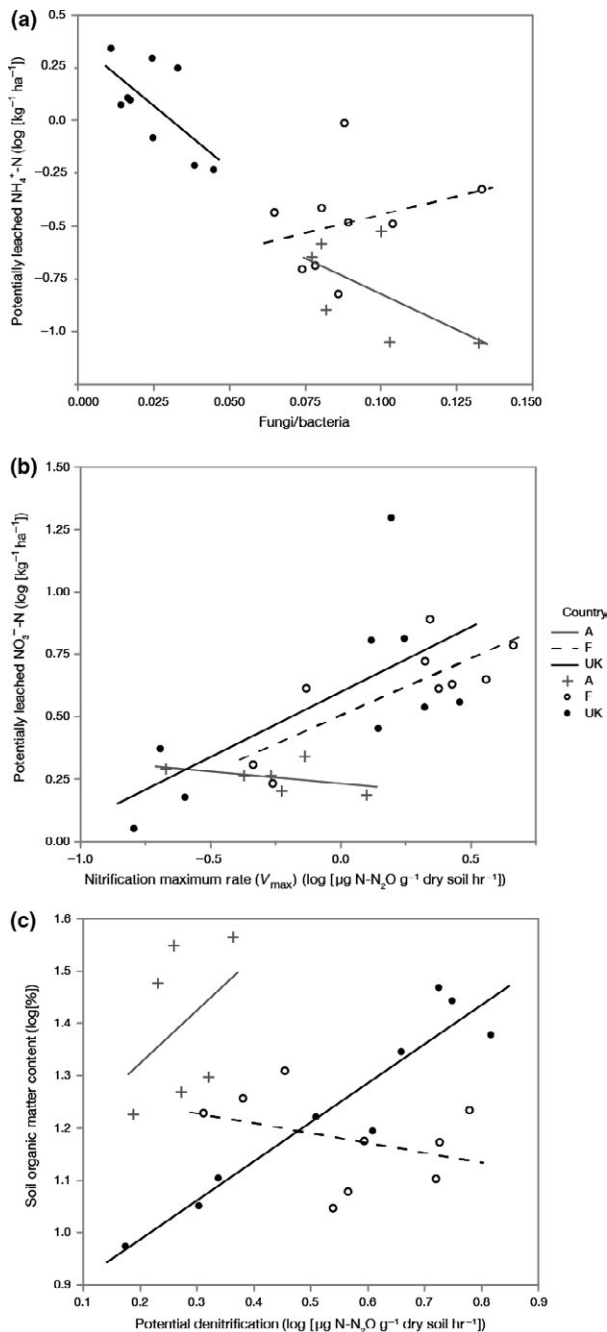
Attributing site as a random factor for the REML approach used to build the SEM consequently hid site-specific links that are also

important to assess when considering microbial functional traits and proxies for ES. Hence, the direct influence of the fungi/bacteria ratio on potential NH<sub>4</sub><sup>+</sup>-N-leaching differed across the three different sites (Figure 3a). Since bacteria (and Archaea) are performing the different steps of nitrification, the F/B ratio should theoretically show a negative correlation with potential NH<sub>4</sub><sup>+</sup>-N-leaching, which was detected in the UK ( $R^2 = 0.43$ ,  $p = .05$ ) and in Austria ( $R^2 = 0.35$ , although not significant) but not in France ( $R^2 = 0.09$ , NS). Similarly, potential NO<sub>3</sub><sup>-</sup>-N-leaching was positively and strongly correlated with the maximum rate of nitrification (Figure 3b) in France ( $R^2 = 0.63$ ,  $p = .001$ ) and in the UK ( $R^2 = 0.45$ ,  $p = .05$ ), but not in Austria ( $R^2 = 0.18$ ,  $p = NS$ ). The strongest influence of site location was observed for the correlation between SOM% and DEA (Figure 3c) which was only significant in the UK site ( $R^2 = 0.9$ ,  $p < .0001$ ).

## 4 | DISCUSSION

### 4.1 | Improving ES understanding with microbial functional traits

This study contributes to the numerous reports of above-ground and below-ground interactions on grasslands ecosystem functioning (Bardgett, Bowman, Kaufmann, & Schmidt, 2005; De Vries et al.,



**FIGURE 3** Site specificity of direct correlations between soil potential for  $\text{NH}_4^+$ -N-leaching and fungi/bacteria ratio (a), soil potential for  $\text{NO}_3^-$ -N-leaching and maximum rates of nitrification enzyme activity ( $V_{\max}$ ) (b) and soil organic matter content and denitrification enzyme activity (c). Grey crosses, white circles and black dots indicate the values transformed to respect normality for Austria, France and UK, respectively. Grey, dashed and black lines indicate the linear correlation between these values for Austria, France and the UK, respectively

2012; Legay, Baxendale, et al., 2014; Orwin et al., 2010; Wardle et al., 2004). Moreover, our results also indicate that direct and indirect interactions between plant and microbial functional traits advance our understanding of the maintenance of soil fertility and potential for N-leaching in managed European grasslands. Following

a more general perspective, several authors have shown a negative correlation between richness, diversity (assessed by bacterial and fungal ribosomal gene analyses) and multiple different ecosystem functions, including net primary productivity (Delgado-Baquerizo, Giamrida, et al., 2016; Delgado-Baquerizo, Maestre, et al., 2016). More specifically here, we demonstrated that including key microbial functional traits in well-defined structural equation models (SEM) significantly improves our ability to explain variance in three proxies of two ES across grassland sites. We acknowledge though that sample size is an important parameter to validate SEM (Shipley, 2013) and authors applying such approach in psychology may even recommend more than 20 observations for each parameters to be estimated using SEM (Wolf, Harrington, Clark, & Miller, 2013). For an analogous reason, a full structural equation (such as the one presented in Figure 1) was not tested nor envisaged, and we considered instead smaller models for specific links between proxies and microbial traits. In total, our approach included 96 experimental units, which may be seen as too little to warrant SEM validity. Although not as good as including more observations, the robustness test indicated that our SEMs were above significance level in more than 80% of the simulations.

Based on *a priori* knowledge, these SEMs revealed that measuring nitrifiers abundances, maximum rates of nitrification, potential denitrification and fungi/bacteria ratios were relevant to better assess potential leaching of mineral forms of N and SOM content in grasslands. Our approach seems more stringent than the proposed correlative approach to estimate genomic traits from the presence/absence of 16S rDNA genes in large dataset from automatically annotated genomes of cultured strains (Barberán et al., 2014; Delgado-Baquerizo, Maestre, et al., 2016), although in some particular cases, phylogenetically related ribosomal genes may show similar functional potential (Amend et al., 2016). Also, measuring microbial functional traits together with above-ground and below-ground plant traits have proven to be well adapted to predict N turnover in grasslands (Cantarel et al., 2015; Legay et al., 2016; Moreau et al., 2015) although with less success in tropical forests harbouring more intermingled roots (Barberán et al., 2015).

The relative contribution of microbes to ES should also deserve a critical synergies/trade-off assessment. Considering the maintenance of soil fertility, the process of nitrification, which involves the oxidation of ammonium to nitrate, could be considered a service or disservice, depending on plant affinity for ammonium or nitrate. It is acknowledged that changes in the  $\text{NO}_3^-$ -N/ $\text{NH}_4^+$ -N ratio strongly influence forage quality and grassland species composition (Maire, Gross, da Silveira Pontes, Picon-Cochard, & Soussana, 2009). Similarly, denitrification, when reduction of nitrate into gaseous forms of N occurs, could be considered as detrimental for soil fertility, but beneficial to plants and soil ecosystems by removing toxic compounds such as nitrite or nitrite oxides. Although we did not measure gaseous output in this study, denitrification should also be considered when assessing the ES of climate regulation because it could release  $\text{N}_2\text{O}$  and  $\text{NO}_x$  gases that have strong greenhouse effect potentials (Pfeiffer & Kaplan, 2010). A significant literature on the (de)nitrification inhibition

is available, although, to our knowledge, it has never been assessed from the final perspective of ecosystem (dis-)services.

## 4.2 | Assessing the relative contribution of microbial traits to specific ES in agroecosystems and considering geographic variation

The stepwise modelling approach that was primarily used when assessing this dataset allowed deciphering the relative contributions of plants and microbes to various ecosystem properties (Grigulis et al., 2013), but it overlooked the potential links between ES and key functional traits of microbes. Overall, while biomass production was strongly linked to plant functional parameters (including plant functional diversity), other ecosystem properties such as carbon sequestration and nutrient retention were mostly driven by microbial processes. Here, we furthermore emphasized which microbial traits were related to these proxies of nutrient retention ( $\text{NO}_3^-$ -N and  $\text{NH}_4^+$ -N-leaching) and C sequestration (%SOM). Hence, the final SEMs retained in our study displayed that these microbial functional traits (especially (de)nitrification parameters, and fungi/bacteria ratio) were relevant to better assess potential leaching of mineral forms of N and SOM content in contrasting grasslands ecosystems, in terms of climate and soil type as well as land management, fertilization and vegetation composition.

Though subalpine meadows and grasslands may not fully represent agroecosystems in structure and function, they do represent a comparable ecosystem in which to study N dynamics that may relate to grass-based agroecosystems. When considering the management of soil fertility and water quality, two ecosystem metrics linked to nutrient retention, land managers operating on systems subjected to higher impact of human activities (e.g. croplands, wetlands) ought to better acknowledge the microbial components and evaluate their relative contributions. Fine-tuning the use of fertilizers could benefit from a better assessment of microbial factors. Hence, since plant-microbe competition for  $\text{NH}_4^+$ -N is strongly linked to its availability in soil (Skiba, George, Baggs, & Daniell, 2011), our results suggest to monitor its concentration as well as the ratio between fungi and bacterial biomasses to link them with PMN. Similarly, because nitrification maximum rate may efficiently drive nitrate availability and potentially to its leaching, measuring the abundances of nitrifiers and considering community-weighted above-ground and below-ground plant traits would bring essential understanding of how herbaceous cover would fully benefit from inorganic fertilization. Finally, because denitrifying microbes are anaerobic-facultative heterotrophs, their dependencies on carbon sources from plant (providing SOM%) and redox conditions (hence to soil moisture and texture) ought to be assessed when considering nutrient retention and soil fertility.

Importantly, the correlations of key microbial variables with proxies of ES showed here geographic specificity, or even opposite trends from the retained models. For instance, nitrate leaching showed an opposite trend, that is, weak negative correlation, with maximum rate of nitrification in the Austrian site, compared

to the two others. This could be related to the particularly low maximum rate of nitrification measured in the Stubai valley, with very little variance among sites. Similarly, the French site showed opposite trends than in the UK and in Austria regarding the correlations between potentially leached  $\text{NH}_4^+$ -N and the fungi/bacteria ratio. This geographic disparity may be related to the fact that compared to the two others sites, the French grassland sites harboured AOA abundances 2.5 logs higher than in the UK and in Austria. AOAs are recognized as main contributors to nitrification in alpine grasslands (Fuchslueger et al., 2014) and may use more efficiently available  $\text{NH}_4^+$ -N. The lack of congruence between SOM% and DEA in the French site is most likely due to the low variance in the measured SOM% among these experimental units, likely related to very low fertilization from local farming (Lamarque et al., 2011).

These geographic specificities underline that considering the correlations between above-ground and below-ground traits described in previous analyses may not be sufficient to avoid the systematic measure of key microbial variables that impacts ES. Despite rapid advances in our ability to evaluate microbial community composition and function, a key applied aspect of the field of microbial ecology remains to be addressed. Specifically, refining techniques for data products that can be easily interpretable by managers (in terms of their influence on ES) represents a key opportunity for improving ecologically base management of soils and other ES in managed agroecosystems.

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## AUTHORS' CONTRIBUTIONS

All authors conceived the ideas and designed methodology. T.P., N.L., C.B., F.P. and J.C.C. collected the data. T.P., A.C., K.G., N.L., F.P. and J.C.C. analysed the data. T.P. led the writing of the manuscript. J.C.C., N.L., R.B. and M.B. significantly improved the quality of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

## DATA ACCESSIBILITY

All data are available under the CCA 3.0 Unported License at the PANGAEA Data Archiving & Publication at the following <https://doi.pangaea.de/10.1594/PANGAEA.879537> (Pommier et al., 2017).



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## SUPPORTING INFORMATION

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