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► To cite this version:

N. Legay, C. Baxendale, K. Grigulis, U. Krainer, E. Kastl, et al.. Contribution of above- and below-ground plant traits to the structure and function of grassland soil microbial communities. *Annals of Botany*, 2014, 114 (5), pp.1011-1021. 10.1093/aob/mcu169. hal-01688359

HAL Id: hal-01688359

<https://hal.science/hal-01688359v1>

Submitted on 4 Sep 2024

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Contribution of above- and below-ground plant traits to the structure and function of grassland soil microbial communities

N. Legay^{1,*}, C. Baxendale², K. Grigulis^{1,3}, U. Krainer⁴, E. Kastl⁵, M. Schloter⁵, R. D. Bardgett⁶, C. Arnoldi¹, M. Bahn⁴, M. Dumont⁷, F. Poly⁷, T. Pommier⁷, J. C. Clément¹ and S. Lavorel¹

¹Laboratoire d'Ecologie Alpine, UMR CNRS 5553, Université Joseph Fourier, Grenoble, France, ²Lancaster Environment Centre, Lancaster University, Lancaster LA1 4YQ, UK, ³Station Alpine Joseph Fourier, UMS 3370 CNRS – Université Joseph Fourier, BP 53, 2233 Rue de la Piscine, 38041 Grenoble Cedex 9, France, ⁴Institute of Ecology, University of Innsbruck, Sternwartestrasse 15, A-6020 Innsbruck, Austria, ⁵Research Unit for Environmental Genomics, Helmholtz Zentrum München, Deutsches Forschungszentrum für Gesundheit und Umwelt (GmbH), Ingolstädter Landstr. 1, 85764 Neuherberg, Germany, ⁶Faculty of Life Sciences, Michael Smith Building, The University of Manchester, Oxford Road, Manchester M13 9PT, UK and ⁷Laboratoire d'Ecologie Microbienne, Université Lyon1, Université de Lyon, 15 USC INRA 1364, UMR CNRS 5557, Villeurbanne Cedex, France

* For correspondence. E-mail nicolas.legay@gmx.com

Received: 23 April 2014 Returned for revision: 30 May 2014 Accepted: 3 July 2014 Published electronically: 13 August 2014

• **Background and Aims** Abiotic properties of soil are known to be major drivers of the microbial community within it. Our understanding of how soil microbial properties are related to the functional structure and diversity of plant communities, however, is limited and largely restricted to above-ground plant traits, with the role of below-ground traits being poorly understood. This study investigated the relative contributions of soil abiotic properties and plant traits, both above-ground and below-ground, to variations in microbial processes involved in grassland nitrogen turnover.

• **Methods** In mountain grasslands distributed across three European sites, a correlative approach was used to examine the role of a large range of plant functional traits and soil abiotic factors on microbial variables, including gene abundance of nitrifiers and denitrifiers and their potential activities.

• **Key Results** Direct effects of soil abiotic parameters were found to have the most significant influence on the microbial groups investigated. Indirect pathways via plant functional traits contributed substantially to explaining the relative abundance of fungi and bacteria and gene abundances of the investigated microbial communities, while they explained little of the variance in microbial activities. Gene abundances of nitrifiers and denitrifiers were most strongly related to below-ground plant traits, suggesting that they were the most relevant traits for explaining variation in community structure and abundances of soil microbes involved in nitrification and denitrification.

• **Conclusions** The results suggest that consideration of plant traits, and especially below-ground traits, increases our ability to describe variation in the abundances and the functional characteristics of microbial communities in grassland soils.

Key words: Ammonia-oxidizing archaea, bacteria, denitrifiers, leaf traits, root traits, nitrite reducers, nitrite oxidizers, nutrient availability, plant functional traits, soil microbial community.

INTRODUCTION

In recent years, plant functional trait approaches have emerged as a powerful tool for identifying the mechanisms by which plants are related to ecosystem processes via their interactions with microbial communities (Fig. 1). There is evidence for such plant trait effects at scales ranging from the individual plant to the landscape (Orwin *et al.*, 2010; de Vries *et al.*, 2012; Grigulis *et al.*, 2013), although their quantification remains a challenge. Grigulis *et al.* (2013) provided a first quantitative estimation of the relative importance of above-ground plant traits and microbial effects for ecosystem nitrogen (N) cycling in grasslands (Fig. 1, grey boxes and arrows). Although some rare studies have analysed plant traits–soil microbe interactions in laboratory conditions in the absence of soil effects (A. Cantarel, Lyon, France, unpubl. res.), similar quantification of other components of plant–soil–microbe interactions in the field is still

required, including the respective effects of plant traits and soil abiotic properties on microbial parameters (Fig. 1, dashed black arrows).

Numerous inter-relationships of abiotic soil properties with plant and microbial communities have been demonstrated. First, the impact of soil properties on plant traits and some soil microbial functional variables is well established. In particular, it is broadly accepted that soil nutrient availability determines the prevalence of certain plant functional traits, such as specific leaf area (SLA) and leaf N content (LNC) (Grime, 1977; Ordoñez *et al.*, 2009). Nutrient-rich grasslands are dominated by fast-growing exploitative species, characterized by high SLA, LNC (Lavorel and Garnier, 2002) and root N concentration (RNC) (Roumet *et al.*, 2006). Conversely, nutrient-poor grasslands are dominated by slow-growing conservative species, characterized by opposite traits. Soil abiotic properties such as pH, and nutrient and water availability are linked not only to plant

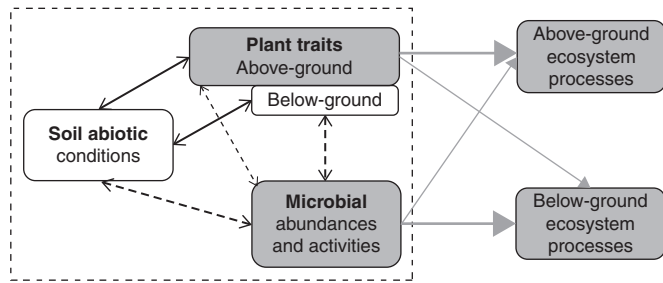


FIG. 1. Schematic diagram showing the links between soil, plants and microbial communities, and their relationships to ecosystem processes. The grey boxes and arrows depict the links found by Grigulis *et al.* (2013). The dashed box and the black dashed arrows represent the links found in the present study.

traits but also to soil microbial communities such as nitrifiers and denitrifiers, and the N turnover processes that they drive (Bremer *et al.*, 2007; Le Roux *et al.*, 2008; Bannert *et al.*, 2011). Second, there is evidence that plants have a pronounced influence on microbial community structure and function, mainly in the rhizosphere or detritosphere. Interspecific differences in plant litter quality have been shown to affect microbial communities and rates of carbon (C) and N mineralization (Wardle, 1992; De Deyn *et al.*, 2008), as have variations in the quantity (Griffiths *et al.*, 1999; Van der Krift *et al.*, 2001; Kuzyakov, 2006) and quality (De Deyn *et al.*, 2008; Henry *et al.*, 2008) of root exudates of individual species. Moreover, recent studies highlight the role of root exudates in providing easily degradable C sources for soil microbes, which stimulates their growth and the mineralization of soil organic matter (Kuzyakov, 2010). In turn, these impacts of plants on soil microbial communities feedback to plant performance, both through their role in modifying nutrient availability and also through various positive or negative interactions involving symbionts and microbial pathogens (van der Heijden *et al.*, 2008; van der Putten *et al.*, 2013).

Despite these known mechanisms, our understanding of multiple relationships among abiotic soil parameters, the soil microbiome and plants remains fragmented, as individual studies have mostly addressed individual associations, making it difficult to build a generic understanding. In addition, most studies have focused on single plant species or cultivars, and have not considered complex species-rich plant communities as found in the field. A few field-based studies have considered the effects of plant functional traits on soil microbes, focusing either on leaf traits alone (Laughlin 2011; de Vries *et al.*, 2012) or specifically on root traits (Valé *et al.*, 2005; Pohl *et al.*, 2011). But to our knowledge, only a few studies have simultaneously considered the effects of above-ground and below-ground plant traits on soil microbial functional variables, such as root decomposition rate or the fungal to bacterial ratio (Orwin *et al.*, 2010; Birouste *et al.*, 2012), and although no direct relationship with root traits were demonstrated, Legay *et al.* (2014) suggested that root systems of exploitative species stimulate selected enzyme activities (e.g. aryl-sulphatase) in soil. Despite this, none of these studies has simultaneously considered how plant traits, both above- and below-ground, and abiotic soil properties explain variation in soil microbial properties under field conditions.

Soil N dynamics in mountain grasslands is typically constrained by low rates of N mineralization and low N inputs (Clément *et al.*, 2012), making N one of the most limiting

nutrients for primary production (Grigulis *et al.*, 2013). Although studies have explored the role of leaf traits as drivers of microbial communities and ecosystem functioning in these grasslands (Orwin *et al.*, 2010; de Vries *et al.*, 2012; Grigulis *et al.*, 2013), no study to date has tested for the relative effects of leaf and root functional traits, and soil abiotic properties, on soil microbial communities and their activities related to N cycling under field conditions. We aimed to bridge this gap using measures at three European mountain grassland sites, where different land-use intensities within each site provided a broad range of variation in plant functional traits, soil properties and microbial functional variables. We focused in this study on the abundance of nitrifiers and denitrifiers as microbial variables, as those microbes strongly influence N availability and quality in soil and thus drive plant performance to a large extent mainly in unfertilized grassland sites. We applied the conceptual and statistical approach developed by Grigulis *et al.* (2013) to quantify the contribution of plant above- and below-ground traits to variation in the gene abundance of functional microbial groups and their resulting activity patterns in grassland ecosystems, once direct effects of abiotic soil properties have been taken into account. We did this by expanding the dataset of Grigulis *et al.* (2013) to include below-ground plant traits and soil abiotic parameters, which allowed us to quantify relative effects of both above- and below-ground traits on microbial functional variables, and assess their importance relative to abiotic effects. We used this approach to test two hypotheses: first, that above- and below-ground plant traits reflecting plant responses to abiotic soil parameters provide unique information about nitrifiers and denitrifiers, which can be considered as important microbial drivers for the inorganic N cycle. This hypothesis was tested by sequentially analysing direct effects of soil abiotic properties on microbial functional variables, and then the additional effects of plant traits. Second, we hypothesized that, whilst above- and below-ground traits may be strongly correlated, root traits are more appropriate for estimating variation in microbial functional variables than the more easily measurable above-ground traits, given the known role of root exudates as drivers of soil microbial communities and their activities. This hypothesis was tested by investigating the relative contributions of above- and below-ground plant traits to the overall effects of plant traits on variations in microbial functional variables.

MATERIALS AND METHODS

Description of field sites

Grasslands were sampled for vegetation and soil properties at three field sites across Europe, at the Lautaret Pass (French Alps), the Stubai Valley (Austrian Alps) and the Yorkshire Dales (UK). These sites were selected to provide a range of grasslands typical of western European upland regions. Moreover, they cover a range of intensities of agricultural activity and their recent changes, including abandonment of management, intensification of grassland production through fertilization and biodiversity restoration for agri-environmental objectives. These sites cover a range of fertilities for semi-natural grasslands, spanning a wide range of N availability and associated vegetation communities (Supplementary Data Table S1). The

past and present management of these different grasslands has been fully described in previous studies (see Grigulis *et al.*, 2013). Briefly, at the French site, three grassland types were chosen along a gradient from medium N availability to poor N availability (Robson *et al.*, 2007): two on terraced slopes, of which one is fertilized and mown for hay in early August, and the second is not fertilized and mown but lightly grazed. The third grassland type is unterraced and only very lightly grazed during travel of livestock to summer pastures. The peak standing green biomass (AGB – Garnier *et al.*, 2007) of these three grasslands types was respectively 4.73, 2.99 and 3.76 tons of dry mass per hectare. At the Austrian site, two grassland types were chosen along an intensity gradient; the first is an intensive meadow with fertilizer application and two or three vegetation cuts a year (AGB = 3.44 t ha⁻¹), and the second is an abandoned meadow which was previously mown and grazed and is now colonized by trees and shrubs (AGB = 2.98 t ha⁻¹). The UK site is characterized by a long-term fertilization gradient. The most intensively managed meadow is subject to high rates of inorganic fertilizer application with 1–2 vegetation cuts per year and heavy inter-season grazing (AGB = 2.71 t ha⁻¹). The second is a meadow with inorganic fertilizer application, a single annual hay cut and inter-season grazing (AGB = 2.44 t ha⁻¹). The third type is a traditionally managed species-rich hay meadow with light inter-season grazing, a single annual hay cut and no fertilizer inputs (AGB = 2.24 t ha⁻¹).

Sampling strategy

Within each site, three replicate fields (100 m²) were sampled for each of 2–3 management types. Within each replicate field, soil and vegetation were sampled at the same time in four fixed quadrats (50 × 50 cm) during peak biomass at the end of June, early July and end of July 2010 for the UK, French and Austrian sites, respectively. Vegetation was cut at the centre of each quadrat (30 × 30 cm) and five soil cores (4.5 cm Ø, 10 cm depth) were taken from each quadrat. Four cores from each of the four corners were sampled simultaneously and subsequently pooled for soil analysis, and a single core was taken from the centre for root trait analyses. One extra core (205 cm³) was sampled next to the root core for measurement of bulk density and parameters related to soil water availability (see next section).

Soil analysis. Fresh composite soil samples from each plot were weighed and passed through a 5.6-mm sieve. Fresh sieved soil was stored at –20°C (for further phospholipid analysis, DNA extraction and quantification of gene abundance) or 4°C and immediately (within 48 h) processed for the determination of microbial biomass and potential enzymatic activities, soil moisture, soil organic matter content, pH, soil texture (using soil subsequently passed through a 2-mm sieve) and soil nutrients [ammonium (soil NH₄⁺), nitrate (soil NO₃⁻), total dissolved nitrogen (TDN), and dissolved organic nitrogen (DON)] from 0.5 M K₂SO₄ soil extracts (Jones and Willett, 2006) using an FS-IV colorimetric chain (OI-Analytical Corp., College Station, TX, USA). *In situ* available soil inorganic N was measured using ion exchange resin bags. In each plot, resin bags were inserted in the soil (5–15 cm deep at a 45° angle) over the growing season at 6-week intervals from May until October

(four times), once over the winter period (November to April), and during the following growing season from May to October 2011 (four times). Resin bags were made using nylon bags (10 × 5 or 5 × 5 cm) containing 5 g of mixed anion cation exchange resin (Amberlite IRN150, VWR International S.A.S., Fontenay-sous-Bois, France). Captured nitrate and ammonium were released from the resins in 1 M KCl and analysed on an FS-IV colorimetric chain (OI-Analytical). Subsamples of fresh soil were dried at 70°C for 1 week to determine soil water content (SWC), followed by 4 h at 550°C to determine soil organic matter content (SOM). 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 1:4 (soil/distilled water) solution. Bulk density and soil porosity were obtained by measuring the dry mass of a fixed-volume (205 cm³) soil core. Prior to drying, 100 mL distilled water was added to saturate each soil core and allowed us to calculate water holding capacity (WHC) and water-filled pore space (WFPS).

Fungal and bacterial biomass were determined using phospholipid fatty acid (PLFA) analysis, using the method of Bligh and Dyer (1959), adapted by White *et al.* (1979) and described by Bardgett *et al.* (1996). This allowed us to calculate the fungal to bacterial PLFA ratio (F/B ratio), which is commonly used as a measure of the composition of microbial communities and has been linked to soil biogeochemical processes (van der Heijden *et al.*, 2008). Microbial biomass nitrogen (N) was measured using the chloroform-fumigation extraction technique of Vance *et al.* (1987).

Kinetic parameters (V_{\max} and K_m) of potential nitrification were measured following a protocol adapted from Koper *et al.* (2010) and Dassonville *et al.* (2011). Potential denitrification enzyme activity (DEA) was measured according to Attard *et al.* (2011). Abundance of nitrifiers [ammonium-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB)] as well as nitrite oxidizers [Nitrospira (NIP) and Nitrobacter (NIB)] were quantified based on the gene copy numbers present in soil of the ammonium monooxygenase gene *amoA* (*amoA*-AOA *amoA*-AOB) and 16S rRNA gene of NIP and *nrxA* gene of NIB, respectively. Abundances of denitrifiers were measured based on the abundance of the nitrite reductase genes [*nirK* (NRK) and *nirS* (NRS)]. Quantitative real-time PCR (qPCR) was done following Grigulis *et al.* (2013). Briefly, soil samples stored at –20°C were used for DNA analyses. 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 Precellys24 Instrument (Bertin Technologies, Montigny-le-Bretonneux, France). Quantity and quality of extracted DNA were tested by spectrophotometry (Nanodrop; PeqLab, Erlangen, Germany). qPCR was carried out using SYBR green as the fluorescent dye. 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).

Plant trait analysis. Vegetation composition was quantified in each replicate field using the Botanal method (Lavorel *et al.*, 2008), and above-ground plant traits [SLA, LNC, leaf C concentration (LCC), leaf C/N ratio, leaf dry matter content (LDMC), height] were measured using standard protocols (Cornelissen *et al.*, 2003). Community-weighted mean traits were calculated

following Casanoves *et al.* (2011). A more detailed protocol of vegetation sampling and above-ground trait measurements is given by Grigulis *et al.* (2013).

Roots were sampled from a dedicated soil core in each of four quadrats per field, which was weighed before careful washing in tepid water to allow separation of roots by floatation using sieve stacks with different mesh, namely 4 mm, 2 mm and 0.2 mm, and separation of live and dead roots was based on visual clues (Bahn *et al.*, 2006). Roots were placed into an alcohol solution (ethanol 10 %, acetic acid 5 %, v/v) and stored at 4°C to maintain freshness until root morphology measurements using digital scanning. Before analysis, roots were suspended in 1 cm of demineralized water in a 29 × 42-cm clear acrylic tray and scanned at 300 d.p.i. with an Epson Expression 10000XL flatbed scanner. Each digital root image was processed using

WINRHIZO software (Regent Instruments Inc., Sainte-Foy-Sillery-Cap-Rouge, Canada) to determine total root length and average root diameter. Roots were then weighed, dried at 70°C and reweighed to calculate root dry matter content (RDMC) and specific root length (SRL). Finally, dry roots were ground to a fine powder for analysis of N and C concentrations. Being obtained from community-level soil cores, these root trait measures represented community-weighted means.

Two additional root properties were calculated, namely total root length and root mass per mass of soil (see the list of abbreviations for plant, soil and microbial variables, as well as their respective category in Table 1).

Data analysis. We used correlative modelling to quantify the respective contributions of soil properties and plant traits to

TABLE 1. Plant traits, and microbial and soil variables measured and their abbreviations

Variable	Abbreviation	Units
Leaf functional traits		
Leaf carbon content	LCC	mg of C g ⁻¹ leaf dry mass
Leaf carbon/nitrogen ratio	Leaf C/N ratio	
Leaf dry matter content	LDMC	mg dry mass g ⁻¹ leaf fresh mass
Leaf nitrogen content	LNC	mg of N g ⁻¹ leaf dry mass
Specific leaf area	SLA	m ² of leaf kg ⁻¹ leaf dry mass
Vegetative height	Vegetative height	cm
Root functional traits		
Root carbon content	RCC	mg of C g ⁻¹ root dry mass
Root carbon/nitrogen ratio	Root C/N ratio	
Root diameter	Root Diameter	mm
Root dry matter content	RDMC	mg dry mass g ⁻¹ root fresh mass
Root length soil mass	RLSM	m of root g ⁻¹ dry soil
Root mass soil mass	RMSM	g of dry root g ⁻¹ dry soil
Root nitrogen content	RNC	mg of N.g-1 root dry mass
Specific root length	SRL	m of root g ⁻¹ root dry mass
Microbial response variables		
Microbial biomass N	MBN	µg N g ⁻¹ dry soil
Fungal to bacterial PLFA ratio	F/B ratio	
Abundance of ammonia-oxidizing Archaea	AOA	no. of gene copies g ⁻¹ dry soil
Abundance of ammonia-oxidizing bacteria	AOB	no. of gene copies g ⁻¹ dry soil
AOA to AOB ratio	AOA/AOB ratio	
Abundance of nitrite oxidizers (<i>Nitrospira</i>)	NIP	no. of gene copies g ⁻¹ dry soil
Abundance of nitrite oxidizers (<i>Nitrobacter</i>)	NIB	no. of gene copies g ⁻¹ dry soil
NIP to NIB ratio	NIP/NIB ratio	
Abundance of the nitrite reductase gene (<i>nirK</i>)	NRK	no. of gene copies g ⁻¹ dry soil
Abundance of the nitrite reductase gene (<i>nirS</i>)	NRS	no. of gene copies g ⁻¹ dry soil
NRK to NRS ratio	NRK/NRS ratio	
Kinetic parameters of potential nitrification (maximal nitrification rate)	V _{max}	µg N-NH ₄ ⁺ g ⁻¹ dry soil h ⁻¹
Kinetic parameters of potential nitrification (ammonium affinity = 1/K _m)	K _m	µg N-NH ₄ ⁺ ml ⁻¹
Potential denitrification enzyme activity	DEA	µg N-N ₂ O g ⁻¹ dry soil h ⁻¹
Soil properties		
Dissolved organic nitrogen	DON	µg N g ⁻¹ dry soil
Soil nitrate	Soil NO ₃ ⁻	µg N-NO ₃ ⁻ g ⁻¹ dry soil
Soil ammonium	Soil NH ₄ ⁺	µg N-NH ₄ ⁺ g ⁻¹ dry soil
<i>In situ</i> nitrate absorbed in resin	NO ₃ ⁻ sorption	µg N-NO ₃ ⁻ g ⁻¹ resin d ⁻¹
<i>In situ</i> ammonium absorbed in resin	NH ₄ ⁺ sorption	µg N-NH ₄ ⁺ g ⁻¹ resin d ⁻²
Soil ammonium to soil nitrate ratio	Soil NH ₄ ⁺ /NO ₃ ⁻ ratio	
Soil organic matter	SOM	%
Soil water content	SWC	%
Total dissolved nitrogen	TDN	µg N g ⁻¹ dry soil
Water filled pore space	WFPS	%
Water holding capacity	WHC	%

Community-weighted mean traits were calculated only for leaf functional traits which were measured at the species level whereas below-ground plant traits were measured directly at the community level.

variation in microbial functional variables. Data were analysed using a hypothesis-driven process, using linear mixed models with restricted maximum likelihood (REML) estimates (Grigulis *et al.*, 2013). Briefly, in our model, grasslands were considered as replicates grouped within each of the three experimental sites (Stubai, Lautaret, Yorkshire Dales). The REML algorithm allows for the specification of the covariance structure induced by such grouping of the data and provides estimations of parameter effects and variance components for both fixed (soil properties and plant traits) and random (site) effects in the model of microbial functional variables. Analyses were carried out using the average information (AI) algorithm to estimate variance parameters using the software package JMP 7.0 (SAS Institute, Cary, NC, USA). All variables were tested for normality, and log transformations applied, as required, prior to analysis. To provide an objective methodology for the selection of the most parsimonious model, analyses were conducted following Díaz *et al.* (2007).

Prior to the REML analyses, the number of variables was reduced using principal component analysis to avoid possible multiple correlations among explanatory variables. 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 colinearity, being removed from the model. This was especially the case for multiple correlated plant traits that form the ‘plant economics spectrum’ (Reich, 2014), but which we decided not to select a priori, or to combine into a single multivariate proxy, to preserve interpretability based on individual traits. Assuming that soil abiotic properties have a larger influence on microbial traits than plant traits (Fierer and Jackson, 2006; de Vries *et al.*, 2012), multi-variable models for each microbial trait were developed by first fitting those soil properties significant in single variable models, and then fitting those plant effects significant in single variable models. Hence, residual plant effects were considered once impacts of soil properties had been accounted for. Final combined models reported variance explained by sites, and once this variance was accounted for, the variance explained by soil properties and by plant traits, respectively. In addition, within plant traits we examined the relative contributions to variance of above- and below-ground traits.

RESULTS

Effect of soil abiotic properties on microbial community structure, abundances and functioning

Five microbial functional variables linked to potential microbial activities (K_m , V_{max}), to gene abundance of nitrifiers (NIP, AOA/AOB ratio) and denitrifier groups (NRS) were related only to soil properties (Table 2). Once site variation was removed, models explained between 25 % (K_m) and 93 % (V_{max}) of their variation. Specifically, except for the AOA/AOB ratio, these microbial functional variables were positively correlated to soil pH. Microbial gene abundances and activities related to nitrification (i.e. AOA/AOB ratio, NIP and V_{max}) were linked to the substrate or products (i.e. NH_4^+ or NO_3^-) of nitrification. Soil pH and total soil C content explained a large proportion of the variation in microbial biomass N (MBN; 97 %) (Table 2).

Effect of plant traits on microbial community structure, abundances and functioning

The addition of plant traits, particularly below-ground traits, increased the explanatory power of models for nine microbial functional variables, namely F/B ratio, AOA, AOB, NIB, NIP/NIB ratio, NRK, NRK/NRS ratio and DEA. The NRK/NRS ratio was the sole microbial property for which only plant traits (root C/N and vegetative height) and no soil properties were retained. All other potential microbial activities and gene abundances were related to combined effects of soil properties and plant traits. The variation explained by this set of parameters varied from 57 % (F/B ratio) to 90 % (microbial biomass N) (once site variation was removed), with 3–100 % of that variation explained by plant traits (Table 2). The role of plant traits in explaining the variation of microbial functional variables was present in models for both the nitrification and the denitrification processes, as well as for the F/B ratio. In the following section, we focus on the most relevant models having a strong contribution of plant traits.

Plant traits explained about half of the within-site variation in the F/B ratio, which was strongly and negatively related to the single below-ground trait root diameter (48 % of the 57 % explained by fixed effects). Total soil C concentration and soil DON accounted for the other half of explained within-site variation, showing negative and positive correlations, respectively, with the F/B ratio (Table 2, Fig. 2).

Overall, four of the six models for microbial gene abundances involved in nitrification processes retained plant traits (Table 2). For example, 71 % of within-site variation in the gene abundance of *amoA* from AOB were explained by a model combining a positive relationship with one soil parameter (*in situ* available soil NH_4^+) and negative relationships with two plant traits (RDMC and root length by soil mass) (Table 2). In this model, the addition of plant traits improved the model fit, leading to a total of 75 % of the variance explained compared with 58.5 % with soil properties alone. Likewise, the abundance of NRK, a gene involved in denitrification, showed a strong positive relationship with the root C/N ratio, which contributed 52.7 % of the 74 % of explained within-site variance. In addition, NH_4^+ availability was positively related to NRK and improved model fit from 39 to 74 % (Fig. 3, Table 2). The NRK/NRS ratio, by contrast, was exclusively explained by two plant traits (root C/N ratio and vegetative height) (Table 2). The potential DEA was strongly linked to soil parameters, with total soil C concentration and NH_4^+/NO_3^- explaining most (87 %) of its within-site variation, with LCC explaining the remaining 13 %.

Relative effects of different plant traits

Above-ground plant traits explained more than 50 % of the variation in below-ground properties and traits, once site variation had been removed. Our results showed that, although above- and below-ground plant traits were both retained in models, they differed in their contributions to variation in microbial functional features. In fact, when retained in models root C/N ratio and RDMC captured a greater proportion of variation of microbial characteristics than above-ground plant traits (Table 2). Hence, the contribution of above-ground plant traits to explained variation ranged from 2.9 to 25 % and was never the best explaining variables. In contrast, the contribution of below-ground plant

TABLE 2. Fixed effects plant trait and soil property variables retained within the multiple variable REML models for each of the microbial properties; also presented is the percentage variation in each microbial property explained by the site, the retained fixed effects, the proportion of explanation afforded by the fixed effects due to plant traits and soil parameters, respectively, and the significance (P), Wald statistic and the direction and magnitude of the standardized effect for each of the retained fixed effects

				% variation explained after site effect was removed				P	Wald effect	Effect	
Response variable	Retained fixed effect	% variation explained by site	% variation explained by fixed effect	Proportion of % variation explained by plant traits	Proportion of % variation explained by soil parameters						
Microbial abundances	Total microbes	Microbial biomass N	pH					<0.0001	41.01	-0.190	
			Total Soil Carbon	59.5	89.8	2.9	97.1	<0.0001	69.22	0.085	
			Specific Leaf Area					0.0012	15.39	-0.020	
	Fungi and bacteria	F/B ratio	Total Soil Carbon	97.6	57.1	47.9	52.1	0.0039	10.95	-0.003	
			Soil DON					0.0375	5.04	0.017	
			Root Diameter					<0.0001	15.29	-0.190	
	Nitrifiers	AOA	pH					<0.001	30.04	0.500	
			Soil NO ₃ ⁻	63.1	78.8	14.6	85.4	0.002	12.99	0.470	
			RDMC					0.0048	10.29	-5.690	
		AOB	NH ₄ ⁺ sorption					<0.001	18.88	0.900	
			RDMC	17.7	74.9	28.8	71.2	0.0265	5.95	-3.350	
			RLSM					0.0325	5.43	-0.530	
		AOA/AOB ratio	Soil total Nitrogen	Soil total Nitrogen	51.5	74.4	0.0	100.0	<0.001	23.91	-1.270
				NH ₄ ⁺ sorption					<0.001	37.36	0.950
				NO ₃ ⁻ sorption					0.0013	17.67	-0.120
				Soil NH ₄ ⁺					0.0164	7.88	0.880
	NIP	pH	Soil NH ₄ ⁺ /NO ₃ ⁻ ratio	24.3	68.7	0.0	100.0	<0.001	20.55	0.428	
							<0.001	26.63	-0.600		
Denitrifiers	NIB	NO ₃ ⁻ sorption					0.023	6.57	0.400		
		RDMC	6.9	62.1	11.4	88.6	0.042	4.92	-4.930		
	NIP/NIB ratio	NH ₄ ⁺ sorption	Leaf C/N ratio	42.1	58.4	24.3	75.7	<0.001	19.04	-1.060	
			NRK					0.0248	6.22	0.036	
	NRS	pH	NH ₄ ⁺ sorption	55.1	74.4	52.7	47.3	0.0112	8.18	0.258	
Root C/N ratio							<0.001	28.86	0.017		
NRK/NRS ratio			41.7	56.6	0.0	100.0	<0.001	25.59	0.385		
Microbial activities	Nitrification	V _{max}	Root C/N ratio	31.3	79.2	100.0	0.0	<0.001	47.37	94.480	
			Vegetative Height					0.0074	18.93	87.410	
			pH					<0.001	63.28	0.300	
	Denitrification	K _m DEA	NO ₃ ⁻ sorption	13.3	93.3	0.0	100.0	0.0197	6.93	0.081	
			Soil NH ₄ ⁺ /NO ₃ ⁻ ratio					<0.001	49.75	-0.457	
			pH	25.1	25.0	0.0	100.0	0.0231	5.97	0.248	
			Total Soil Carbon					<0.001	78.44	0.006	
			Soil NH ₄ ⁺ /NO ₃ ⁻ ratio	30.2	85.0	13.0	87.0	<0.001	26.28	-0.160	
			Leaf Carbon Content					0.0008	15.94	-0.006	

RLSM (root length per gram of dried soil), C/N ratio (carbon/nitrogen content ration), RDMC (root dry matter content), DON (dissolved organic nitrogen), NO₃⁻ and NH₄⁺ sorption (*in situ* nitrate and ammonium absorbed in resin), F/B ratio (fungal to bacterial PLFA ratio), AOA and AOB (ammonia-oxidizing archaea and bacteria, respectively), NIP and NIB for nitrite oxidizers (genera *Nitrospira* and *Nitrobacter*, respectively), NRK and NRS (nitrite reductase genes), V_{max} and K_m (kinetic response variables of potential nitrification), DEA (potential denitrification enzyme activity).

traits ranged from 11.4 to 75 % and represented the best category of explaining variables in two models as compared with above-ground plant traits or soil properties (Table 2).

DISCUSSION

Overall, our findings indicate the importance of incorporating both above- and below-ground plant traits, in addition to soil

abiotic properties, into models explaining variations in soil microbial functional variables associated with N cycling (Fig. 4, size of dashed black arrows). Indeed, both plant compartments explained significant and independent components of variation in soil microbial community composition and functional variables across the broad range of grasslands studied, pointing to their significance as drivers of microbial communities. Also, although several above- and below-ground plant traits were correlated (Table 3), as expected from previous studies (Freschet *et al.*,

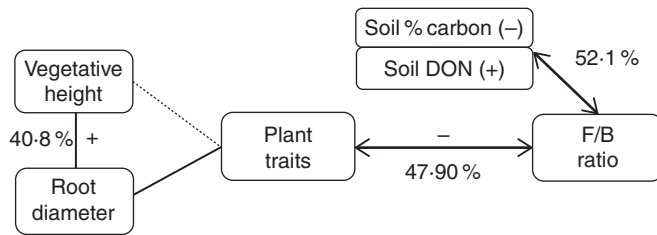


FIG. 2. Schematic diagram showing potential links and percentages of explanation of soil properties and plant traits on the F/B ratio (fungal to bacterial PLFA ratio). Positive and negative correlations are represented by + and – symbols next to the arrows when only one variable was involved in the relationship, and by (+) and (–) next to each variable when two variables were involved in the relationships.

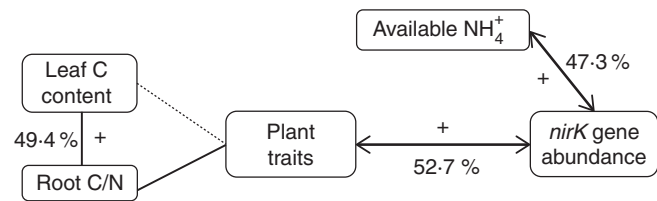


FIG. 3. Schematic diagram showing potential links and percentages of explanation of soil properties and plant traits on NRK gene abundance. Positive and negative correlations are represented by + and – symbols.

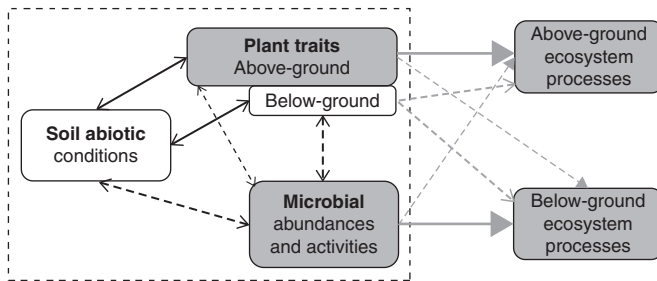


FIG. 4. Schematic diagram showing the potential mechanisms by which soil and plants are related to microbial communities and ecosystem processes. The grey boxes and arrows depict the links in Grigulis et al. (2013) and the dashed box represents the links modelled in the present study. The size of the dashed black arrows is proportional to the relative contribution of soil abiotic properties, and above- and below-ground plant traits on variance of microbial functional variables. The dashed grey arrows extrapolate the potential changes that the present study could bring to the contribution of plant and microbial communities on ecosystem processes described by Grigulis et al. (2013).

2010; Birouste et al., 2012), below-ground traits retained in models captured a greater proportion of variation in measured microbial properties than did leaf traits (Table 2).

Effects of soil abiotic properties on microbial response variables

Abiotic soil properties (pH and nutrient availability) constituted major drivers of soil microbial activities and abundances, as demonstrated in previous studies (e.g. Fierer and Jackson, 2006; Leininger et al., 2006; de Vries et al., 2012). Soil pH was strongly related to many microbial variables (e.g. NEA, AOA, NIP, MBN) and, when retained in models, this parameter had the largest contribution to total explained variation, which is consistent with the findings of previous studies (Fierer and

Jackson, 2006; Eskelinen et al., 2009; Laughlin, 2011). Logically, soil nutrient availability (NH_4^+ and NO_3^-), which influences microbial growth and activity, also strongly contributed to variation in microbial functional characteristics (Henry et al., 2008; Attard et al., 2010; Verhamme et al., 2011; Le Roux et al., 2013). In contrast, although soil water availability was tested in single or multiple REML models among other explanatory variables for each of the microbial measurements (Supplementary Data Table S2), it was never retained in final models, which was presumably due to its correlation with soil N or ammonium concentrations (data not shown).

Effects of plant traits on microbial processes

To the best of our knowledge, no other study has linked both above- and below-ground plant traits to both soil microbial activities and microbial gene abundances associated with N cycling in field conditions. The inclusion of plant traits significantly improved statistical models for response variables relevant to nitrification and denitrification. Moreover, for one measure, the NRK/NRS ratio, plant traits were the only parameters retained in the final model, and for two measures, F/B ratio and NRK, root traits predicted a large proportion of the variation.

A few studies have highlighted significant effects of plant traits on soil microbial biomass and activities at the individual plant, ecosystem and landscape scale (Orwin et al., 2010; Laughlin, 2011; de Vries et al., 2012). We found that fine roots (i.e. high SRL), which tend to be less developed in slow-growing plants typical of nutrient-poor environments (Craine et al., 2003), were positively associated with the F/B ratio. This suggests that plant traits of slow-growing species could mediate their effects on the composition of microbial communities in nutrient-poor and/or carbon-rich environments. This is consistent with findings of associations of a higher abundance of fungi relative to bacteria with low community-level SLA (de Vries et al., 2012) or high rooting density of individual species (Orwin et al., 2010). Nevertheless, the link observed between root traits and the relative abundance of fungi and bacteria should be considered with caution given possible over-estimation of fungal biomass due to the potential contribution of root cells to the abundance of polyunsaturated PLFAs such as 18:2 ω 6 (Zelles, 1997; Kaiser et al., 2010), even after sieving and manual removal of root fragments. We also observed a negative relationship between total soil C concentrations and ratio of fungal to bacterial PLFAs, which contrasts with recent findings (de Vries et al., 2012). A plausible explanation for this difference is that a decrease in total soil C could reflect a drop in labile soil C content, which usually favours bacterial communities at the expense of fungal communities (Fontaine and Barot, 2005).

Plant traits contributed to explaining the variation in microbial functional gene abundances, but had low contribution to variation in potential microbial activities (0 % for nitrification and 13 % for denitrification). In fact, all variation in N-related microbial processes was predicted by soil parameters, as they controlled the availability of N-NH_4^+ and N-NO_3^- processed by nitrification and denitrification, and thereby outweighed any direct plant trait effects. The most influential plant trait predicting variation in the abundance of genes involved in nitrification (e.g. AOA, AOB and NIB) was RDMC (Fig. 5), with only root

TABLE 3. Fixed effects leaf trait variables retained within the multiple variable REML models for each of the root properties; also presented is the percentage variation in each root property explained by the site, the retained fixed effects, and the significance (P), Wald statistic and the direction and magnitude of the standardized effect for each of the retained fixed effects

Response variable	Retained fixed effect	% variation explained by site	% variation explained by model (after site effect removed)	P	Effect	Wald
RLSM	LDMC	56.5	34.0	0.025	2.63	5.88
	Leaf N Content			0.043	-0.02	4.71
RMSM	Leaf C Content	66.7	59.0	<0.001	0.54	29.44
	Vegetative Height			0.011	-0.76	14.20
Root Diameter	Vegetative Height	89.1	40.7	<0.001	0.01	14.91
SRL	Leaf C Content	87.0	35.2	0.0023	-0.02	12.14
RDMC	Leaf C Content	30.5	39.9	0.003	0.00	14.03
	Leaf N Content			0.045	0.00	4.54
Root C Content	Leaf C Content	10.6	50.6	<0.001	1.08	17.25
	Vegetative Height			0.005	-1.66	9.85
Root N Content	Leaf C Content	4.5	37.3	0.004	-0.08	10.16
Root C/N ratio	Leaf C Content	2.7	49.3	<0.001	0.48	17.60

RLSM and RMSM (root length and root mass per gram of dried soil), RDMC (root dry matter content), LDMC (leaf dry matter content), C content and N content were the content of carbon and nitrogen in leaves or roots.

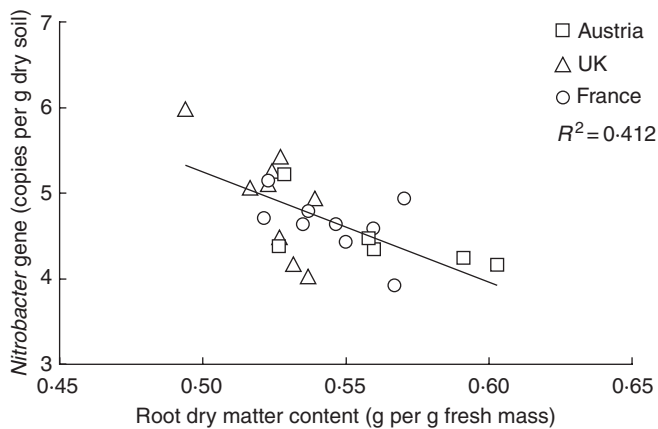


FIG. 5. Relationship between root dry matter content and gene copy numbers of *Nitrobacter*.

length and leaf C/N also being retained in models of the gene abundance of nitrifiers. Such relationships could be mediated by N availability because both RDMC (Craine *et al.*, 2001) and nitrifier gene abundance (Attard *et al.*, 2010; Verhamme *et al.*, 2011) are known to be strongly linked to soil nutrient availability. Grasslands with high N availability are often characterized by exploitative plant communities (e.g. low RDMC) and high N turnover rates (e.g. high nitrification rate and nitrifier abundance) (Robson *et al.*, 2007). Consequently, the negative relationship between RDMC and nitrifier gene abundances may simply result from their simultaneous responses to a change of soil N availability.

Concerning denitrifier gene abundances, two of the three response variables studied (NRK and consequently NRK/NRS ratio) were primarily explained by plant traits in the statistical models (Table 2). In both cases, the root C/N ratio was the most significant variable with an increase of this ratio being related to increased NRK. As root exudate quality impacts on microbial communities (Griffiths *et al.*, 1999), we suggest that root C/N ratio is a proxy of exudate quality and production

(Valé *et al.*, 2005), and notably a proxy of the exudate C/N ratio which directly influences microbial communities (Bremer *et al.*, 2007; Henry *et al.*, 2008). Nevertheless, more studies are needed concerning the quality of root exudates and their assimilation by microbial communities to support this hypothesis. Additionally, root C/N ratio may influence the C/N ratio of labile soil fractions (Fornara *et al.*, 2011), with possible indirect effects on denitrifier abundance (Yin *et al.*, 2002). Hence, these two potential mechanisms suggest that high root C/N ratio might be related to an exudation of high C/N compounds, which could change C/N ratios of labile soil fractions and impact the NRK community (Henry *et al.*, 2008).

Although unlikely because sampling was done in early summer, we cannot exclude an indirect effect of root decomposition on soil C availability and then on denitrifiers. It is also possible that a high root C/N ratio could be either related to low N uptake by plants (Craine *et al.*, 2003), or the result of competition with denitrifiers which could limit plant N uptake and lead to low N in roots. These characteristics are typical plant traits in nutrient-poor environments where soil NO_3^- availability is low, which may also impact the gene abundance of some denitrifiers. Finally, leaf traits had significant effects in models of variation in NRK/NRS ratio and DEA, improving model fit by 25% (with vegetative height) and 13% (with LCC), respectively (Table 2). This suggests an indirect effect of leaf traits on denitrifiers linked to litter quality, as a correlation was shown between leaf litter quality and LCC (Fortunel *et al.*, 2009) but also with vegetative height in one of our study sites (Lavorel and Grigulis, 2012). Litter quality, by driving partly the nature of C sources available for microbes, may impact the gene abundance of microbial communities and their ability to use N resources (Wardle, 1992; Falcão Salles *et al.*, 2012).

Relative importance of plant above- and below-ground traits for variation in microbial functional variables

The relative abundance of microbial genes related to key N-processes were in some cases significantly related to both above- and below-ground plant traits. As detailed above, a

small set of below-ground plant traits (root diameter, RDMC, root C/N ratio) were highly influential in predicting variation in soil microbial community structure across the grassland sites. Likewise, [Orwin et al. \(2010\)](#) found that RNC and root C concentration (RCC), along with root biomass (not a functional trait *per se*), showed stronger relationships to soil community structure (microbial biomass and F/B ratio) than did leaf traits. Consequently, although root and leaf traits are dependent on each other ([Craine et al., 2001](#); [Freschet et al., 2010](#)), they probably ensure distinct effects or relationships with N-related microbial communities and processes. In the present study, five root traits were retained in the models describing the structure, abundances and activities of microbial communities, and it is difficult to identify a specific root trait for studying plant–microbial relationships. However, all of the selected root traits were linked to plant nutrient uptake capacity ([Craine et al., 2003](#)), and were likely to be highly influenced by soil nutrient availability ([Craine et al., 2001](#)), and have been suggested to impact root exudate quantity and quality ([Valé et al., 2005](#)).

The strong contribution of root traits to statistical models of microbial response variables could be related to two potential mechanisms. On the one hand, root traits reflect plant responses to soil parameters including pH and nutrient conditions, with effects on microbial activities and community structure being additional to direct impacts of these soil parameters. On the other hand, root traits determine the amount and the quality of plant C and N supply to soil microbial communities (mainly denitrifiers), which depend on both soil nutrient status and plant nutrient uptake ([Van der Krift et al., 2001](#)). However, the amount of root exudates may also be linked to the strength of the C sink in microbial communities ([Bahn et al., 2013](#)). Therefore, such feedback effects from microbial communities to plant traits should be incorporated for a more complete understanding of complex inter-relationships among soil abiotic properties, plant traits and microbial community structure and activities, especially using experimental approaches ([Harrison and Bardgett, 2010](#)). In the same way, the mycorrhizal status of roots could be included in our analysis as a key root trait, especially given the known roles of mycorrhizal fungi in soil N cycling ([Read and Perez-Moreno, 2003](#); [Veresoglou et al., 2011](#)).

Finally, the contribution of below-ground plant traits to microbial functional variables could change the estimation of the relative contributions of plant and microbial parameters to N-cycling processes ([Grigulis et al., 2013](#)). This assumption is supported by recent studies showing the role of root traits on N-cycling through their link with root litter decomposability ([Birouste et al., 2012](#)) or microbial community composition ([Orwin et al., 2010](#)). By accounting for these effects, the contribution of microbial variables to variations in soil ecosystem properties across grasslands, as studied by [Grigulis et al. \(2013\)](#), could decrease in favour of root traits (Fig. 4, dashed grey arrows).

CONCLUSIONS

Our findings highlight the importance of incorporating plant traits, and especially root traits, in analyses of variation in soil N-related microbial activities and community structure and abundances across a broad range of European mountain grasslands. As expected, we found that soil abiotic properties, including soil pH and nutrient availability, were the primary drivers of

soil microbial functional variables. However, consistent with our first hypothesis, indirect pathways via above- and below-ground plant functional traits explained additional variation in soil microbial community (microbial gene abundances of N-related processes and F/B ratio) across grassland communities. Also, consistent with our second hypothesis, below-ground traits were more closely linked to microbial properties than were leaf traits, suggesting that consideration of root traits, in addition to leaf traits, increases our ability to explain variation in the structural and functional characteristics of microbial communities in grassland soils. Finally, we suggest that future experiments are needed to advance our understanding of the mechanisms involved in those indirect effects from soil abiotic properties to microbial functional variables that operate via plant functional traits.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: study site characteristics. Table S2: results of REML tests of individual soil properties and plant traits as explanatory factors for each microbial property and functional parameter.

ACKNOWLEDGEMENTS

This study was conducted as part of ERA-Net BiodivERsA project VITAL, ANR-08-BDVA-008, with the national funders ANR, FWF, NERC, BMBF and Austrian Science Fund (FWF project number I 242-B17). The Lautaret site is part of the long-term research site Zone Atelier Alpes, a member of the ILTER-Europe and LTSER networks. The sites at Stubai are part of the LTSER platform Tyrolean Alps. We acknowledge the Joseph Fourier Alpine research station (CNRS UMS 3370) for infrastructure support, and Edith Primat, Hanna Secher-Frommell and Marie-Pascale Colace for help with field and laboratory measurements at Lautaret. We thank Nadine Guillaumaud for her support in the field campaign and her help for the measurements of microbial activities on the AME platform (UMR5557-USC1364). qPCR to estimate abundances of *Nitrobacter* and *Nitrospira* were performed at the DTAMB platform (FR41, University Lyon1). We also thank Dario Fornara and an anonymous reviewer for their considerable input in improving the manuscript.

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