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Microbial soil biodiversity in beech forests of European mountains[1]

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Abstract: *Fagus sylvatica* L. is widely distributed across Europe thanks to its high adaptability in a wide variety of soils and climate. Microbial communities are essential for maintaining forest soil quality and are responsible for forest ecosystem functioning; the ability of soil microorganisms to respond to abiotic stressors (e.g., organic carbon losses, water scarcity, temperature changes) is crucial under ongoing environmental changes and also supports tree health. In this study, soil samples were collected from pure beech plots as part of the COST Action project CLIMO to find differences in microbial community characteristics and evaluate the effects of soil properties on microbial communities across gradients of elevation, latitude, and longitude. Positive relationships were found between organic carbon content and both microbial abundance and dehydrogenase activity. Dehydrogenase and catalase activities were elevation-correlated and microbial activities were longitude-correlated. In the most southern beech plot, the microbial community was abundant and displayed high activities. This shows that microbial communities could help tree populations to better adapt to predicted changes in environmental conditions in the future. We suggest that research into forest health and beech performance should also test soil microbial enzymatic activity, particularly under changing climate conditions, to assist in identifying adaptation strategies.

Key words: *Fagus sylvatica*, soil quality, soil enzyme activities, elevational, latitudinal, and longitudinal transects, soil microbial structure.

Résumé : *Fagus sylvatica* L. est largement présent à travers l'Europe grâce à sa capacité élevée d'adaptation à une grande variété de sols et de climats. Les communautés microbiennes sont essentielles au maintien de la qualité des sols forestiers et responsables du fonctionnement des écosystèmes forestiers; la capacité des microorganismes du sol de réagir aux stress abiotiques (p. ex. la perte de carbone organique, la pénurie d'eau, les changements de température) est cruciale face aux changements environnementaux en cours et assure également la santé des arbres. Dans cette étude, des échantillons de sol ont été prélevés dans des parcelles de hêtre en peuplement pur dans le cadre du projet CLIMO, une Action COST visant à trouver des différences entre les caractéristiques des communautés microbiennes et à évaluer les effets des propriétés du sol sur les communautés microbiennes le long de gradients élévational, latitudinal et longitudinal. Des relations positives ont été observées entre d'une part la teneur en carbone organique et d'autre part l'activité de la déshydrogénase et

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l'abondance microbienne. L'activité de la déshydrogénase et de la catalase était corrélée avec l'élévation et l'activité microbienne était corrélée avec la longitude. Dans les parcelles de hêtre les plus méridionales, la communauté microbienne était abondante et très active. Cela indique que les communautés microbiennes pourraient aider les populations d'arbres à mieux s'adapter aux changements prévus dans les conditions environnementales futures. Nous croyons que la recherche sur l'état de santé des forêts et la performance du hêtre devrait également s'intéresser à l'activité enzymatique microbienne dans le sol, particulièrement dans le contexte des changements climatiques, pour aider à identifier des stratégies d'adaptation. [Traduit par la Rédaction]

Mots-clés : *Fagus sylvatica*, qualité du sol, activité enzymatique dans le sol, transects élévational, latitudinal et longitudinal, structure microbienne su sol.

Introduction

Fagus sylvatica L. (European beech) is widely distributed in temperate regions in Europe, because it is sensitive to drought and high temperatures (Colin et al. 2017) and less sensitive to soil conditions (Walthert et al. 2013). Forest ecosystems such as beech stands provide several ecosystem services, including timber production, carbon sequestration, soil fertility, water quantity and quality, and preservation of biodiversity (Duncker et al. 2012). Soil microbial communities are responsible for the functioning, stability, sustainability, and productivity of forest ecosystems (Leckie 2005; van der Heijden et al. 2008; Defossez et al. 2011; Mercado-Blanco et al. 2018; Grosso et al. 2018; Manzanedo et al. 2018). Their enzyme activities play key roles in carbon cycling in ecosystems by controlling important processes such as litter decomposition, nutrient cycling, plant diversity, nitrogen fixation, and plant nutrient uptake (Lladó et al. 2017). The complex interactions between trees and soil microbial communities in beech forests are poorly understood (Nacke et al. 2016). Nevertheless, the effect of soil microbial communities on biogeochemical cycles and key processes in beech forest soils together with their spatial variations is poorly studied (Nacke et al. 2016). In this context, elevational and latitudinal transects offer the opportunity to study the distribution of microbial communities through the natural gradients of soil conditions and of various climate regimes, as well as the presence or absence of trees within short distances (Ma et al. 2004; Körner 2007; França et al. 2016; Siles and Margesin 2017; Kotas et al. 2018). These transects could also be useful for studying soil microbial structure and activity in forest stands, including beech forests (Yergeau et al. 2006; Grayston and Rennenberg 2006; Wu et al. 2009; Zhang et al. 2013; Cardelli et al. 2019).

Environmental condition changes with elevation have been reported to influence soil microbial communities (Zhang et al. 2013). A decrease in fungal biomass and diversity with increasing elevation was observed in the Austrian Alps (Margesin et al. 2009). A negative correlation between bacterial abundance and elevation was reported by Ma et al. (2004) in the cold, temperate Kalasi Lake and by Giri et al. (2007) in a dry, tropical deciduous forest. By contrast, Margesin et al. (2009) found several shifts in microbial community composition with increasing elevation in Alpine soils such as a significant increase in the relative abundance of fungi and gram-negative bacteria (see also Zhang et al. 2013). Other studies (Männistö et al. 2007; Shen et al. 2013) reported that elevation-induced changes in microbial community composition are controlled by pH rather than by temperature fluctuations in Arctic fjelds in Finnish Lapland and on Mount Changbai. Moreover, Djukic et al. (2010) reported that microbial community structure is connected with decomposition conditions and changes in vegetation composition along an elevation gradient in the Austrian Alps (on limestone). These studies suggest that soil microbial communities cannot be easily predicted and are linked to a wide range of factors in addition to elevation (Zhang et al. 2013).

The enzymatic activity of the soil microbial community has been used to evaluate soil health and different soil functioning such as decomposition rates, and in some cases, these activities were found to be lower at the lowest elevations (Jang and Kang 2010). De Feudis et al. (2017) highlighted that enzymatic activity is not affected by elevation, whereas Margesin et al. (2009) demonstrated that soil dehydrogenase activity decreases with elevation and that it is lower in cold Alpine soils than in subalpine soils. In the latter study, an increase in the relative number of cultivable psychrophilic heterotrophic bacteria, fungi, and gram-negative bacteria was found with increasing elevation. These studies show that the level of enzymatic activity can vary with edaphic gradients and environmental conditions.

The COST Action Climate-Smart Forestry in Mountain Regions (CLIMO) (http://climo.unimol.it) has been working to identify and harmonize climate-smart forestry key indicators and management options in the European context (Tognetti 2017; Santopuoli et al. 2021). The Action is linked with the EU regulations for integrating emissions and removals from land use, land-use change, and forestry (LULUCF) into the EU Climate and Energy framework (Nabuurs et al. 2017). An agreed definition of climate-smart forestry and the process for selecting indicators to assess the "climate smartness" of forest management have been recently provided by Bowditch et al. (2020). Their definition combines the adaptation, mitigation, and social dimensions of climate-smart forestry and has paved the way for developing and selecting indicators in support of the implementation of climate-smart forestry in Europe (Bowditch et al. 2020).

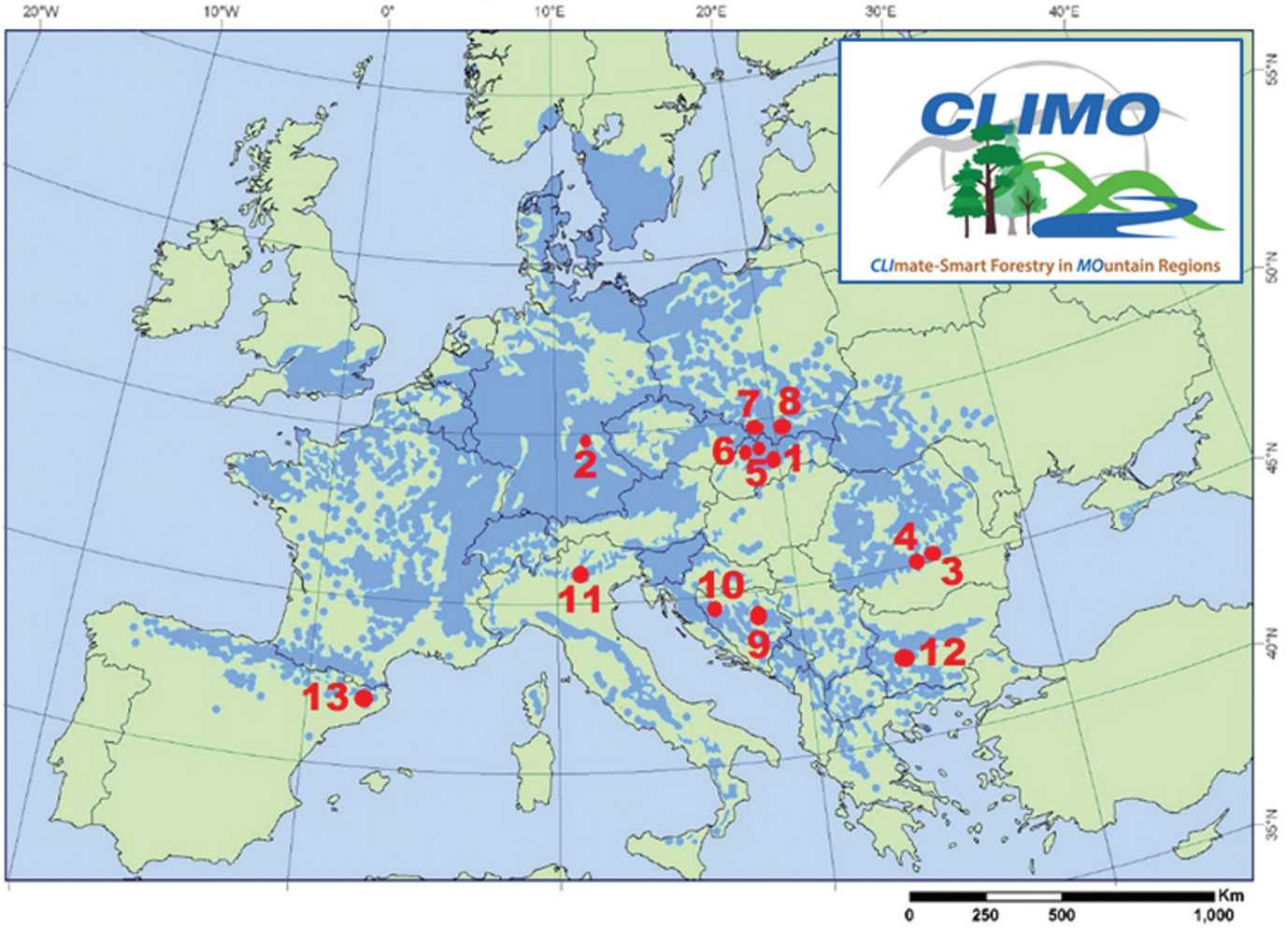
The aim of this study was to evaluate variability in microbial communities in the soils of European beech forests in mountain regions along latitudinal, longitudinal, and elevational gradients. We used the soil samples collected from the European network of permanent plots established across pure mountain European beech forests within the COST Action CLIMO. The permanent plots selected are located at different elevations (400–1400 m a.s.l.), latitudes (from Spain to Poland), and longitudes (from Spain to Romania). The structure and activity of the autochthonous microbial communities were evaluated at two soil depths (0–15 and 15–30 cm). In particular, the microbial abundance, dehydrogenase, catalase, and urease activities, and the main microbial groups (assessed using ester-linked fatty acids) were analyzed. The data were evaluated by considering abiotic factors such as pH, organic carbon, total nitrogen, organic and total phosphorus contents, elevation, and latitude as potential predictors of soil microbial activity and structure. We examined the following hypotheses. (1) Is microbial community composition in beech stand soils at different elevations, longitudes, and latitudes affected by abiotic factors? (2) Does the relative abundance of decomposer microorganisms decrease with soil depth? (3) Do microbial communities respond more to longitudinal than latitudinal gradients? (4) Does the activity of microbial communities decrease with increasing elevation?

Materials and methods

Study areas, soil sampling, and processing

For the present study, 13 pure beech permanent plots from eight European countries (Spain, Italy, Germany, Slovakia, Romania, Bulgaria, Poland, and Bosnia) considered in the COST Action CLIMO in 2017 were selected at different elevations (Fig. 1). Table 1 reports the locations of the permanent plots and the main characteristics of the soils; brief descriptions of the sampling sites are

Fig. 1. Location of the selected pure beech (*Fagus sylvatica*) plots: 1, Slovakia A (SVK A); 2, Germany (DEU); 3, Romania A (ROU A); 4, Romania B (ROU B); 5, Slovakia B (SVK B); 6, Slovakia C (SVK C); 7, Poland A (POL A); 8, Poland B (POL B); 9, Bosnia A (BIH A); 10, Bosnia B (BIH B); 11, Italy (ITA); 12, Bulgaria (BGR); and 13, Spain (ESP). Beech stand distribution areas in Europe are in blue. The base map is from the European Forest Genetic Resources Programme (EUFORGEN, http://www.euforgen.org), modified with Adobe Photoshop. [Colour online.]



reported in Supplementary material S1[2]. The sampling of soils was performed by removing the surficial stratum (litter, fermentation, and humus layers, 2–5 cm), which may have contained leaves or grass. Although soil sampling has to take into consideration soil horizons (Cools and De Vos 2016), the soil type at all plots was Cambisol (except for the Italian plot, which was a Leptosol; see Table 1), and the soil depths were >40 cm. Consequently, the two sampling depths (0–15 and 15–30 cm) were selected by assuming that the first 15 cm comprise the main soil stratum where all microbial activities take place (Grayston and Rennenberg 2006; Cong et al. 2015). The soil sampling was performed in October–November 2018. Mean annual, summer, or autumn temperatures and precipitation in the selected areas are reported in Supplementary Tables S1 and S2[2].

To obtain representative soil samples from each beech plot at two depths, the samplings were performed in accordance with European harmonized methods (Cools and De Vos 2016). In particular, five sampling points in the same beech plot (situated 10 m away from each other) were sampled at the two depths (0–15 and 15–30 cm) and mixed to obtain two composite samples (1 kg each). These were transported in sterile boxes kept at 4 °C to the CNR-IRSA Institute (Italy) for some of the analyses (pH, microbial abundance, ester-linked fatty acids (ELFA), organic carbon, total nitrogen and phosphorus contents) and to Oradea University (Romania) for the total number of fungi and dehydrogenase, catalase, and urease activities. Each analysis was performed three times, with three sub-aliquots from each sample.

Soil properties

The measurement of the soil reaction (pH) was performed in 1:2.5 soil–water suspensions (Conyers and Davey 1988), using a pH meter (pH 50+ DHS, XS Instruments, Italy). Specifically, approximately 5 g of soil was dissolved in 12.5 mL of distilled water shaken at 35.8g for 2 h and then allowed to settled for 24 h. Organic carbon (C_{org}) and total nitrogen contents were measured using a CHNS analyzer (Carlo Erba NA 1500 series 2 C/H/N/O/S, Milan, Italy). Two air-dried soil subsamples (15–20 mg) for each soil sample were sieved with a 0.5 mm sieve, acidified (20 μL of 5 mol·L^{-1} ultrapure HCl), and kept at 50–60 °C for 30 min to remove inorganic carbon. Organic carbon content and nitrogen content are expressed as percentages (%) of dry mass.

[2]Supplementary data are available with the article at https://doi.org/10.1139/cjfr-2020-0139.

Table 1. Main characteristics of the sampling sites.

No.	Plot name	Acronym	Latitude (N)	Longitude (E)	Elevation (m a.s.l.)	Exposure	Slope (°)	Geological characteristics	Soil type
1	Slovakia A	SVK A	48.67796667	19.47016667	1180	N	10	Andezite	Andic cambisol
2	Germany	DEU	49.08566111	13.30653056	1120	SW	3	Granitic	Cambisol
3	Romania A	ROU A	45.495830	25.18777778	1461	NW	10	Limestone	Eutric cambisol
4	Romania B	ROU B	45.53722	25.883815	1277	NE	25	Conglomerates and quartzite	Eutric cambisol
5	Slovakia B	SVK B	49.17146667	19.08181667	767	NW-W	32	Devonian limestone	Cambisol
6	Slovakia C	SVK C	49.28516667	16.73927778	490	E	3	Devonian limestone	Cambisol
7	Poland A	POL A	49.62243056	18.91460278	520	SW	22	Clay mixed with rocks	Cambisol
8	Poland B	POL B	49.43298333	20.903100	830	SW	20	Magura sandstone	Hyperdystric cambisol
9	Bosnia A	BIH A	43.724444	18.28583333	1290	N-NW	14	Fluvio-glacial sandstone and limestone	Calcic cambisol
10	Bosnia B	BIH B	44.64408611	16.66843333	524	E-NE	4	Limestones and dolomite	Calcic cambisol
11	Italy	ITA	46.11888889	12.42972222	1090	NE	5	Limestone moraine	Leptosoil
12	Bulgaria	BGR	42.672500	23.85083333	1350	W-NW	25	Sandstone	Cambisol
13	Spain	ESP	41.77555556	2.45666667	1186	S	18	Granit and granodiorite	Dystric cambisol

Total and organic phosphorus (total P and organic P) contents were determined by applying a modification of the ignition procedure by Saunders and Williams (1955); the analyses were conducted using the colorimetric molybdenum blue method by Olsen and Sommers (1982). The soil samples were first frozen at −20 °C and then lyophilized (to prevent any influence of soil moisture on the measurements). To measure total P, soil samples (1 g in three sub-replicates) were ashed in a muffle furnace (1 h, 550 °C). These samples together with three other non-ashed sub-replicates (1 g each, used for the inorganic P) were incubated with 50 mL of H_2SO_4 (0.5 mol·L^{-1}) and shaken for 16 h at 5g at room temperature. The samples were filtered (0.22 μm phosphate-free filter) and neutralized. Eight millilitres of a reactive solution (6 g of $(NH_4)_6Mo_7O_{24} \times 4H_2O$ and 0.1454 g of $K_2(SbO)_2C_8H_4O_{10} \times 3H_2O$, dissolved in 1 L of H_2SO_4, 1.25 mol·L^{-1}) and ascorbic acid solution (1 mL, 42.24 g·L^{-1}) were added to an aliquot of the filtered samples (10 or 5 mL for inorganic P or total P, respectively). A final volume of 50 mL was then reached with distilled water. After 30 min, absorbance (at 893 nm) was measured with a Perkin-Elmer Lambda 40 UV/VIS spectrophotometer. Quantification of phosphorus was obtained with calibration curves in the range of 0–1 μg P·mL^{-1}. The amount of P in the soil (micrograms of P per gram of soil) was calculated as $[(S \times 50\ \text{mL})/(\text{number of grams of sample} \times F)]$, where S is the average phosphorus content in the samples (μg P), and F is the filtered volume (5 or 10 mL). Organic P represents the difference between total P and inorganic P. All the soil properties were measured using 2 mm sieved soil samples (except for organic carbon and total nitrogen determinations).

Microbial analysis of soil samples

Before the analyses, the soil samples were dried for 2–3 days at room temperature, the impurities (plant residues, insects) were eliminated, and the samples were then sieved (2 mm). To evaluate the dehydrogenase activity and to quantify the fungi, the soil samples were dampened and incubated for 5 days at 37 °C.

The total microbial abundance (number of cells per gram of dry soil) was assessed in the freshly sampled soils with the epifluorescence direct count method, using 4′,6-diamidino-2-phenylindole (DAPI) as the DNA fluorescent dye (Barra Caracciolo et al. 2005). DAPI allows microbial cells (which appear in a luminescent blue colour) to be distinguished from non-living bacterium-sized particles (which appear yellow) under an epifluorescence microscope. Briefly, the soil samples (1 g, three sub-replicates) were fixed with 9 mL of a fixing solution (phosphate-buffered saline: 130 mmol·L^{-1} NaCl; 7 mmol·L^{-1} Na_2HPO_4; 3 mmol·L^{-1} NaH_2PO_4; 2% formaldehyde (*v*/*v*); 0.5% Tween 20 (*v*/*v*); and 100 mmol·L^{-1} sodium pyrophosphate). The test tube was shaken (15 min, 35.8g), and then left at 4 °C for 24 h so that the larger soil particles could settle. An aliquot of supernatant (100–300 μL) was transferred into a sterile tube containing 2 mL of sterilized physiological solution with 200 μL of DAPI. After 30 min in the dark, the solution was filtered through a 0.2 μm black membrane that was subsequently mounted on a glass microscope; the microbial cells were counted using an epifluorescence microscope (DM LB30, Leica GmBH, Heideberg, Germany).

The microbial community structure was evaluated using the ELFA method to obtain a phenotypic fingerprint of the main microbial groups (gram-positive and gram-negative bacteria, fungi) occurring in the different soil samples. The extraction method, performed in accordance with that described by Schutter and Dick (2000) and Di Lenola et al. (2018), uses a mild alkaline reagent to lyse cells and release fatty acids from lipids as methyl esters. Only ester-linked and non-free fatty acids were extracted. Briefly, 3 g of soil (fresh mass) was mixed with 15 mL of 0.2 mol·L^{-1} KOH in methanol and 3 μg of internal standard (C19:0); it was then shaken at 2.2*g* at 37 °C for 1 h, enabling the release and subsequent methylation of the ELFAs. Acetic acid (3 mL, 1.0 mol·L^{-1}) was then added to neutralize the pH, and the extraction of fatty acid methyl esters (FAMEs) was performed using 10 mL of hexane. The upper layer containing hexane was evaporated in a desiccating centrifuge for 1 h and then resuspended in 100 μL of hexane. Finally, the analytical determinations were performed with gas chromatography (GC) coupled to a flame ionization detector (FID) (Clarus 480, Perkin Elmer), using a DB-5MS (Rtx-5MS, 30 m length × 0.32 mm i.d. × 0.25 μm film thickness; Restek) capillary column, following the method reported elsewhere (Rauseo et al. 2019). Fatty acid peaks were identified by comparing the peak retention times in unknown samples with those of fatty acid methyl esters (FAME, cat. no. 47885-U, standard solution in dichloromethane, Supelco) and bacterial acid methyl esters standards (BAME, cat. no. 47080-U, standard solution in methyl caproate, Supelco). The relative abundance of fatty acids found in the different soil samples was expressed as mole percent (mol %) of total fatty acids. The ELFA results corresponding to fungi (18:1ω9c and 18:2ω6,9c), gram-negative bacteria (16:1ω7, 18:1ω7c, and 19:0cy), gram-positive bacteria (i15:0, a15:0, i16:0, and i17:0), total bacteria (15:0, i15:0, a15:0, i16:0, i17:0, 17:0, 16:1ω7, 18:1ω7c, and 19:0cy), and *Actinomycetales* (10Me16:0, 10Me17:0, and 10Me18:0) were used as signature biomarkers for these microbial groups (Bossio and Scow 1998; Zelles 1999). Results are reported as molar percentages (% mol) of total fatty acids.

The total number of fungi was evaluated via the plate count method, using the Sabouraud agar culture medium on decimal dilutions of soil samples, with 0.5% chloramphenicol (pH 5.4) (Dehghan et al. 2014). The Petri plates containing the inoculated culture media were incubated (4–5 days of incubation at 25 °C) in a thermostatic oven (UNB 100, Memmert, Germany). The fungi colonies were then counted using a colony counter (LKB 2002,

POL EKO, Poland). The values obtained were multiplied by the dilution factor (10^4), resulting in the number of units capable of forming colonies (CFU) per gram of soil (Bölter et al. 2002).

Enzymatic analyses of dehydrogenase and catalase activities were performed to measure soil microbial metabolism and activity; urease activity was conducted for soil nitrogen metabolism determination. Actual and potential dehydrogenase activity (ADA and PDA, respectively) were determined according to the methods described by Casida et al. (1964). The reaction mixtures consisted of 3.0 g soil, 0.5 mL 2,3,5-triphenyltetrazolium chloride (TTC), and 1.5 mL distilled water or 1.5 mL glucose solution for ADA or PDA, respectively. All the reaction mixtures were incubated at 37 °C for 24 h. After incubation, the triphenylformazan produced was extracted using acetone and was measured spectrophotometrically at 485 nm. Dehydrogenase activity was expressed as milligrams of triphenylformazan (TPF) produced (from TTC) by 10 g of soil in 24 h.

Catalase activity (CA) was determined using the permanganometric method (Drăgan-Bularda 1983). The reaction mixtures consisted of 3.0 g soil, 2 mL H_2O_2 3%, and 10 mL phosphate buffer. The incubation was performed at 37 °C for 1 h. CA was expressed as mg H_2O_2 decomposed by 1 g of soil in 1 h.

Urease activity (UA) was determined using the method described by Kandeler and Gerber (1988). The reaction mixtures consisted of 5.0 g soil, 5 mL buffer solution, and 5 mL substrate solution. Reaction mixtures without soil or without substrate were used as controls. All the reaction mixtures were incubated at 37 °C for 2 h. The ammonium released was extracted using potassium chloride solution and measured spectrophotometrically at 445 nm. The results are reported as mg NH_4 per 100 g soil in 2 h.

To establish a hierarchy of the plots, giving equal importance to the four enzymatic activity measurements, the enzymatic indicator of soil quality (EISQ) was calculated as follows (Muntean et al. 1996):

$$\text{EISQ} = \frac{1}{n} \times \sum_{i=1}^{n} \frac{Vr(i)}{V\max(i)}$$

where n is the number of enzymatic activity measurements, $Vr(i)$ is the real individual value of the activity, and $V\max(i)$ is the maximum theoretical individual value of the activity.

The maximum individual values, calculated from the composition of the reaction mixtures, were 13.45 mg triphenylformazan (from 15 mg TTC in the initial reaction mixture) for the ADA and PDA, 60 mg H_2O_2 (the quantity added to the initial reaction mixture) for the CA and 85 mg ammonium (from 150 mg urea in the initial reaction mixture) for the UA. The EISQ can range between 0 (when there is no activity in the studied samples) and 1 (when the real individual values are equal to the maximum theoretical individual values for all enzymatic activity measurements) (Samuel et al. 2017).

Statistical analysis

We used a t test to evaluate differences in each biotic parameter (total microbial abundance, activities, and structure measured by the ELFA method), C_{org}, total N contents, and pH between the sample depths.

The correlations among elevation, latitude, and longitude and the different biotic factors (microbial abundance, different activities, and various bacterial groups and fungi) were assessed with the Statistica Software package (www.tibco.com/products/tibco-statistica) and MS Excel to find correlations between biotic and abiotic factors.

An overall rank analysis of data was also performed as a preliminary test to visually identify in a table the samples that differed from others. A heat map chart was used for this purpose, using different colours to represent data values in a table. Due to the wide range in values across multiple variables, with large differences in magnitude and different measurement units, a rank (from 1 for the highest level to 26 for the lowest) was given to all the results (gram-positive and gram-negative bacteria, fungi percentage, total nitrogen and C_{org} contents, soil moisture, total microbial number, pH, PDA, ADA, CA, and UA, and total and organic P content) as a standardization of the data. The total rank of each sample was then calculated (sum of the rank of each parameter, i.e., rank for total nitrogen plus rank for total microbial number, etc.) and the chart was constructed.

Finally, the principal component analysis (PCA) of all the data (with longitude, latitude, and elevation factors) was conducted with R (version 3.4.4; www.r-project.org) to identify patterns and to highlight the similarities and differences between different soil samplings. A PCA analysis of all sampling points (0–15 and 15–30 cm depth) was performed considering the following factors: microbial abundance; elevation; pH; C_{org}; ratio of fungi to bacteria (fungi–bacteria ratio); ratio of nitrogen to phosphorus (N:P), and EISQ. Before the PCA analyses, the dataset was standardized by mean and standard deviation in MS Excel.

Results

Physicochemical analyses

Soil pH ranged from 4.10 to 6.65, with no statistically significant differences between surface and deep soil layers at the same site (Supplementary Table S3[2]). The soils with an acidic pH (<5.5) were POL A and B, SVK B and C, ROU A and B, ITA, and BGR (Fig. 2).

The C_{org} and total N (Figs. 3 and 4; Supplementary Table S3[2]) detected in the surface samples (0–15 cm) were always higher than the concentrations detected in the samples at deeper depth at the German site (DEU), Slovakian sites (SVK A, SVK B, and SVK C), and Polish sites (POL A and POL B) (paired t test, $p < 0.05$) but not significant for the others. The highest values of C_{org} were found in ESP samples at both depths (7.56% and 7.03% for 0–15 and 15–30 cm, respectively).

Organic P ranged from 168.19 to 1609.79 $\mu g \cdot g^{-1}$ soil, with the highest values ($p < 0.01$) for ROU A (Fig. 5). The N:P ranged from 0.60 to 8.80, with the highest values ($p < 0.01$) found in ESP (Supplementary Table S3[2]). These values indicate that the amount of phosphorus is not a limiting factor in these soils (Xu et al. 2019).

Enzymatic activity measurements and correlations with elevation, latitude, and longitude and with soil chemical properties

The results for the different enzymatic activities and the EISQ are reported in Table 2. As expected, in general, the values were higher in surface soil samples (0–15 cm) than in deeper ones (15–30 cm). The EISQ value varied from 0.506 to 0.94 (Table 2), with the highest values found for the Spanish and Slovakian soils.

None of the correlations between elevation and the enzymatic indicators analyzed was significant when the samples from both depths were merged (data nor shown); however, significant correlations (direct or inverse) were found between elevation and ADA at 0–15 cm ($r = 0.432$; $p < 0.05$) and between elevation and CA at 15–30 cm depth ($r = -0.326$; $p < 0.05$) (Supplementary Table S5[2]). ADA and PDA increased together with the elevation of the permanent plots, while CA decreased. Correlations between enzymatic activities and latitude were not significant (Supplementary Table S5[2]), except for ADA for the surface samples.

Finally, a significant correlation ($p < 0.01$) was found between CA and both pH and the N:P (Supplementary Table S6[2]) and between UA and organic carbon.

Microbial communities and correlations with elevation, latitude, and longitude

The total microbial number (Fig. 6; Supplementary Table S3[2]) at the 15–30 cm soil depth (mean value: $5.91 \times 107 \pm 1.22 \times 10^7$ cells$\cdot g^{-1}$) was lower than or comparable with that detected at the 0–15 cm

Fig. 2. Soil pH measured at each sampling point and depth (0–15 and 15–30 cm) of the pure beech plots. Sampling points: Slovakia A, B, and C (SVK A, SVK B, and SVK C, respectively); Germany (DEU); Romania A and B (ROU A and ROU B, respectively); Poland A and B (POL A and POL B, respectively); Bosnia A and B (BIH A and BIH B, respectively); Italy (ITA); Bulgaria (BGR); and Spain (ESP). The vertical bars represent the standard errors. [Colour online.]

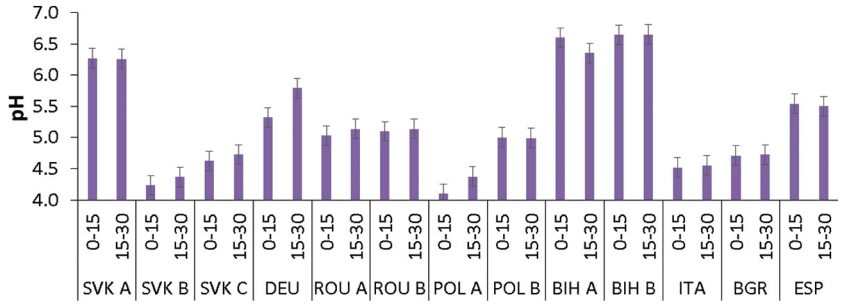


Fig. 3. Soil organic carbon found at each sampling point and depth (0–15 and 15–30 cm) of the pure beech plots. Plots: Slovakia A, B, and C (SVK A, SVK B, and SVK C, respectively); Germany (DEU); Romania A and B (ROU A and ROU B, respectively); Poland A and B (POL A and POL B, respectively); Bosnia A and B (BIH A and BIH B, respectively); Italy (ITA); Bulgaria (BGR); and Spain (ESP). The vertical bars represent the standard errors. [Colour online.]

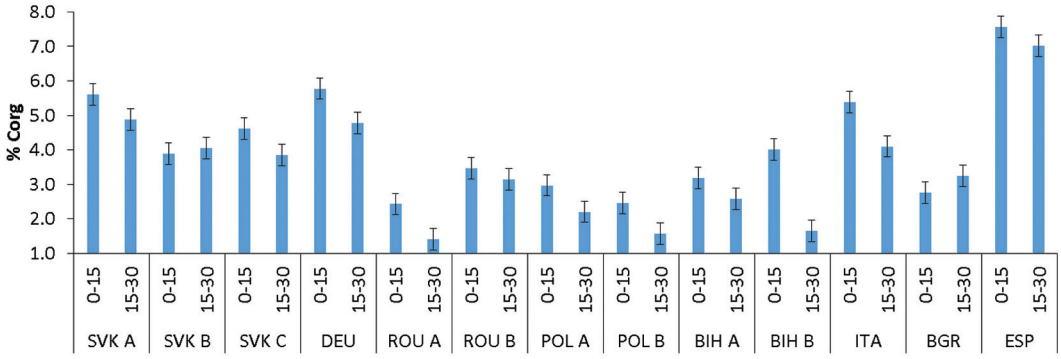
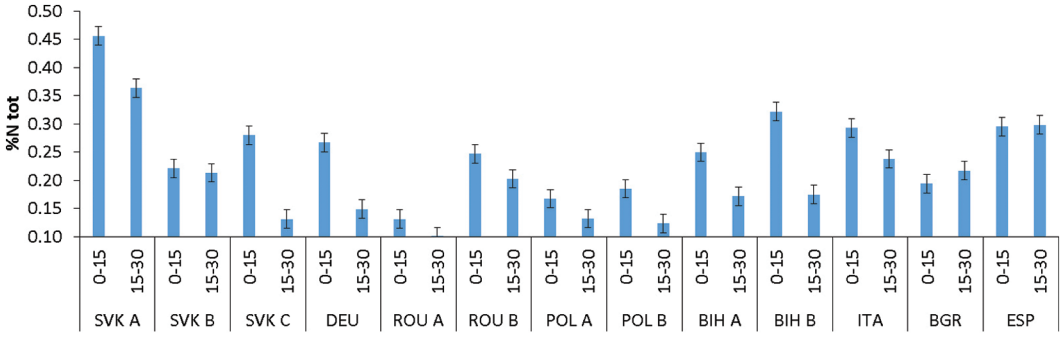


Fig. 4. Soil total nitrogen found at each plot and depth (0–15 and 15–30 cm) of the pure beech plots. Sampling points: Slovakia A, B, and C (SVK A, SVK B, and SVK C, respectively); Germany (DEU); Romania A and B (ROU A and ROU B, respectively); Poland A and B (POL A and POL B, respectively); Bosnia A and B (BIH A and BIH B, respectively); Italy (ITA); Bulgaria (BGR); and Spain (ESP). The vertical bars represent the standard errors. [Colour online.]



soil depth (mean value: $1.38 \times 10^8 \pm 2.08 \times 10^7$ cells·g^{-1}) in all the samples analyzed. The situation was similar for gram-negative bacteria (Fig. 7; Supplementary Table S3[2]) and for bacteria and fungi measured with the ELFA method (Fig. 8), where the upper 15 cm of soil displayed higher values than those of the corresponding deeper samples. As regards gram-positive bacteria (Fig. 7; Supplementary Table S3[2]), the values were high in some samples (0–15 cm POL A, 15–30 cm > ESP > BIH B 0–15 cm), but there was not a clear distinction between shallower and deeper soil layers.

Unlike bacteria, the number of fungi (Supplementary Table S3[2]) was generally higher in the deeper samples than in the upper samples. The only exceptions were found in the Slovakian and Spanish samples in which the values were similar.

In the case of *Actinomycetales* (Fig. 9; Supplementary Table S3[2]), with a few exceptions (e.g., POL A and B, BIH A and B), the trend was similar to those reported for fungi, which was that the deeper samples contained higher numbers than the shallower samples.

Fig. 5. Total and organic phosphorus found at each plot and depth (0–15 and 15–30 cm) of the pure beech plots. Sampling points: Slovakia A, B, and C (SVK A, SVK B, and SVK C, respectively); Germany (DEU); Romania A and B (ROU A and ROU B, respectively); Poland A and B (POL A and POL B, respectively); Bosnia A and B (BIH A and BIH B, respectively); Italy (ITA); Bulgaria (BGR); and Spain (ESP). The vertical bars represent the standard errors. [Colour online.]

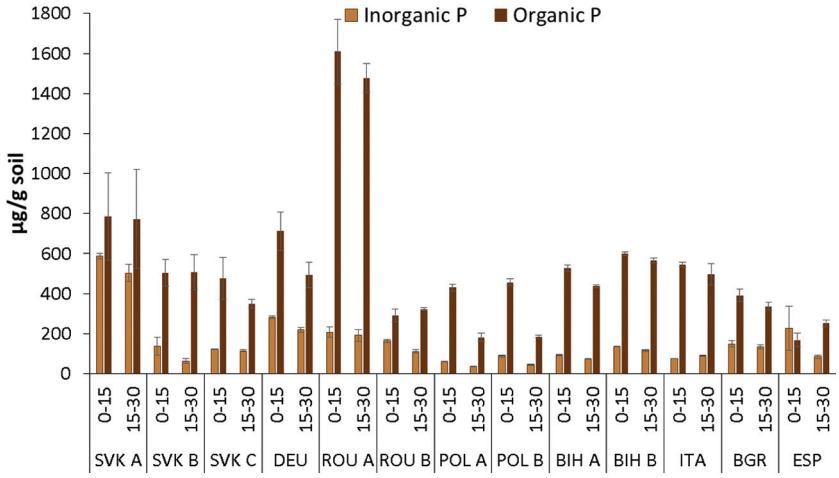


Table 2. Soil actual and potential dehydrogenase, catalase, and urease activities and the enzymatic indicator of the soil quality in the permanent plots of European beech forests within the COST Action project CLIMO.

Plot	Depth (cm)	ADA (mg TPF per 10 g soil per 24 h)	PDA (mg TPF per 10 g soil per 24 h)	CA (mg H_2O_2 per g soil per h)	UA (mg NH_4 per 100 g soil per 2 h)	EISQ
SVK A	0–15	14.28	15.23	8.1	5.614	0.659
	15–30	5.46	10.13	6.8	3.498	0.506
DEU	0–15	12.29	15.06	7.2	5.980	0.635
	15–30	12.51	13.63	7.4	4.481	0.698
ROU A	0–15	16.29	26.18	9.6	3.098	0.632
	15–30	14.00	18.42	9.5	2.948	0.675
ROU B	0–15	17.02	28.50	9.2	3.698	0.728
	15–30	11.53	13.63	8.9	2.598	0.615
SVK B	0–15	11.81	18.67	11.6	4.548	0.687
	15–30	7.67	17.86	10.5	3.998	0.715
SVK C	0–15	17.75	33.54	11.8	5.397	0.898
	15–30	14.28	26.09	11.2	4.581	0.925
POL A	0–15	12.34	23.26	9.4	3.365	0.687
	15–30	12.04	17.52	9.5	2.665	0.724
POL B	0–15	13.07	22.90	9.5	3.991	0.665
	15–30	13.41	14.75	9.4	2.982	0.683
BIH A	0–15	23.35	27.13	9.3	3.598	0.780
	15–30	15.23	18.34	9.2	2.932	0.732
BIH B	0–15	12.96	16.96	9.3	2.615	0.559
	15–30	9.40	12.43	9.4	2.449	0.579
ITA	0–15	9.35	16.04	10.9	5.131	0.652
	15–30	8.87	13.38	10.1	4.498	0.709
BGR	0–15	20.72	32.56	9.2	3.881	0.804
	15–30	13.41	27.52	9.4	3.915	0.850
ESP	0–15	24.52	25.70	12.2	4.414	0.875
	15–30	18.95	25.45	11.2	3.848	0.940

Note: ADA, actual dehydrogenase activity; PDA, potential dehydrogenase activity; CA, catalase activity; UA, urease activity; EISQ, enzymatic indicator. Sampling points: Slovakia A (SVK A); Germany (DEU); Romania A (ROU A); Romania B (ROU B); Slovakia B (SVK B); Slovakia C (SVK C); Poland A (POL A); Poland B (POL B); Bosnia A (BIH A); Bosnia B (BIH B); Italy (ITA); Bulgaria (BGR); Spain (ESP).

There were no significant correlations between total microbial abundance and latitude or elevation, although higher r values were found in the case of latitude (Supplementary Table S4[2]).

The microbial community composition and activities were not correlated with elevation if both the soil depths were considered. Significant correlations were recorded between elevation and ADA in the upper soil layer and between elevation and CA in the deeper soil layer (in the latter case, the correlation was negative). Both ADA and PDA increased with elevation, while CA decreased (Supplementary Table S5[2]).

Overall rank analysis

The overall rank analysis (Tables 3 and 4) was extremely useful in the identification of "outlier" samples. It was possible to estimate the extent to which a single sample differed from the others. Samples from Spain, Slovakia, Germany, and Italy

Fig. 6. Microbial community: total microbial abundance in the soil samples of the pure beech plots at the two depths (0–15 and 15–30 cm). Sampling points: Slovakia A, B, and C (SVK A, SVK B, and SVK C, respectively); Germany (DEU); Romania A and B (ROU A and ROU B, respectively); Poland A and B (POL A and POL B, respectively); Bosnia A and B (BIH A and BIH B, respectively); Italy (ITA); Bulgaria (BGR); and Spain (ESP). The vertical bars represent the standard errors. [Colour online.]

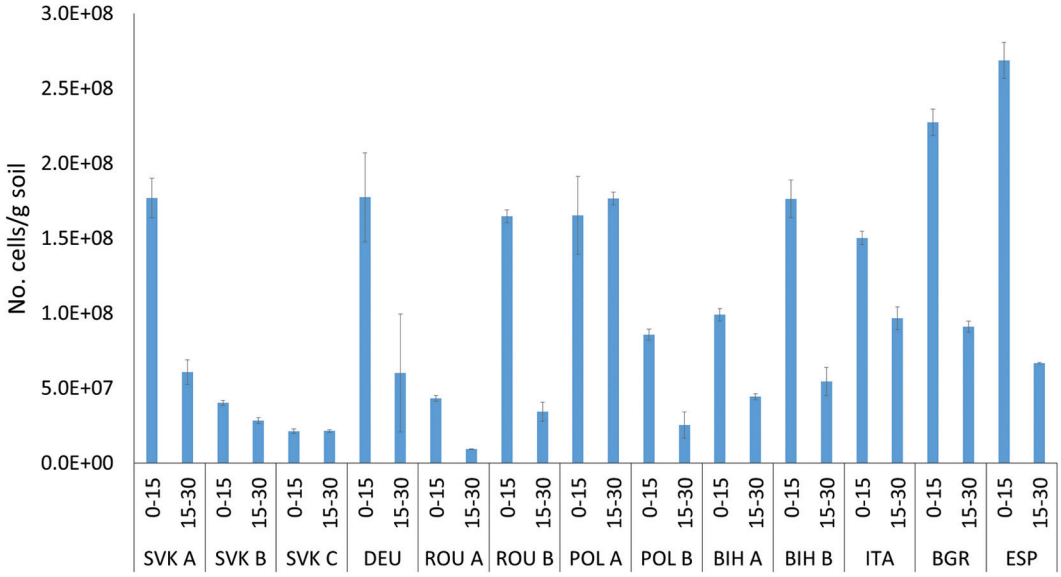


Fig. 7. Microbial community structure (gram-positive and gram-negative bacteria; fungi), evaluated with the ester-linked fatty acid (ELFA) method, at each sampling point of the pure beech plots and depth (0–15 and 15–30 cm). Results are expressed as relative abundance of fatty acids (mole percent, mol %) found in the different soil samples. Sampling points: Slovakia A, B, and C (SVK A, SVK B, and SVK C, respectively); Germany (DEU); Romania A and B (ROU A and ROU B, respectively); Poland A and B (POL A and POL B, respectively); Bosnia A and B (BIH A and BIH B, respectively); Italy (ITA); Bulgaria (BGR); and Spain (ESP). The vertical bars represent the standard errors. [Colour online.]

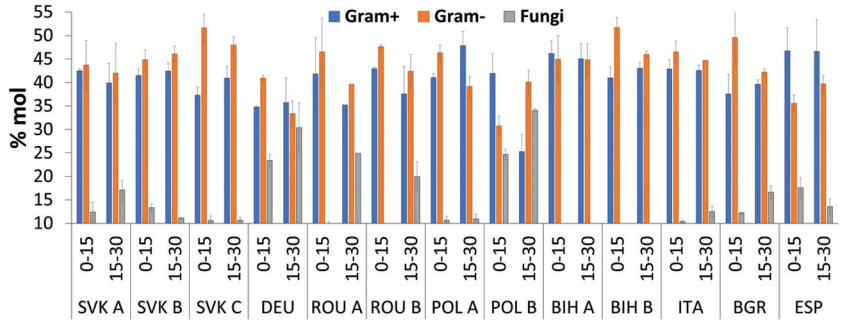


Fig. 8. Comparison between bacteria and fungi (evaluated with the ELFA method) in each soil sample and depth (0–15 and 15–30 cm) of the pure beech plots. Results are expressed as relative abundance of fatty acids (mole percent, mol %) found in the different soil samples. Sampling points: Slovakia A, B, and C (SVK A, SVK B, and SVK C, respectively); Germany (DEU); Romania A and B (ROU A and ROU B, respectively); Poland A and B (POL A and POL B, respectively); Bosnia A and B (BIH A and BIH B, respectively); Italy (ITA); Bulgaria (BGR); and Spain (ESP). The vertical bars represent the standard errors. [Colour online.]

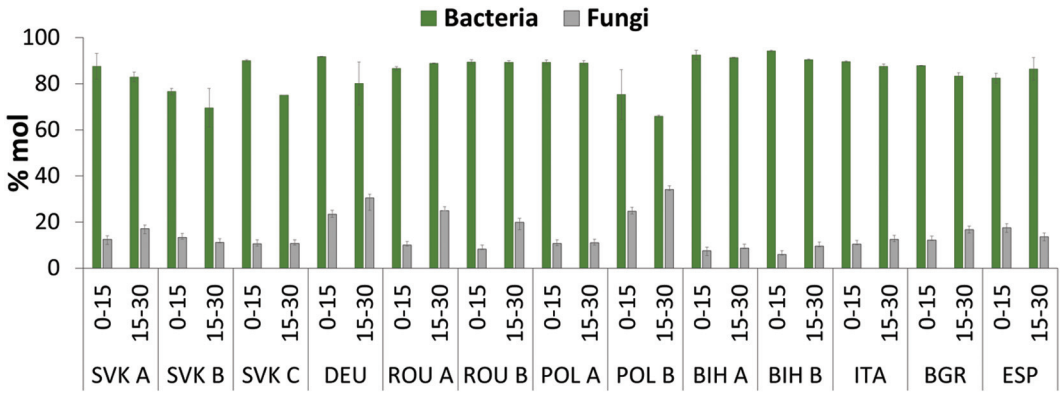


Fig. 9. Actinomycetales (evaluated with the ELFA method) in each soil sample and depth (0–15 and 15–30 cm) of the pure beech plots. Results are expressed as relative abundance of fatty acids (mole percent, mol %) found in the different soil samples. Sampling points: Slovakia A, B, and C (SVK A, SVK B, and SVK C, respectively); Germany (DEU); Romania A and B (ROU A and ROU B, respectively); Poland A and B (POL A and POL B, respectively); Bosnia A and B (BIH A and BIH B, respectively); Italy (ITA); Bulgaria (BGR); and Spain (ESP). [Colour online.]

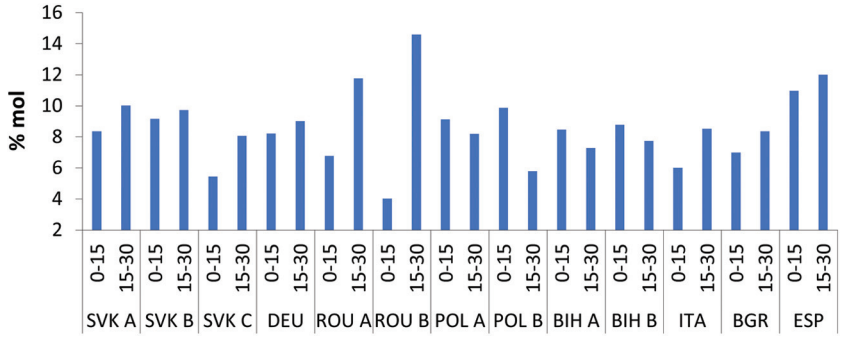


Table 3. Heat map chart representing the overall rank of each sample. [Colour online.]

Total rank	SVK A	DEU	POL A	POL B	SVK B	SVK C	ROU A	ROU B	BIH A	BIH B	ITA	BGR	ESP
UP	89.5	126	173.5	166	155	123	173	127	140.5	131.5	112	155	77
DOWN	149	139	179	205	160	150	204.5	182.5	167	174.5	130	167	104.5
Overall (UP + DOWN)	238.5	265	352.5	371	315	273	377.5	309.5	307.5	306	242	322	181.5

Note: Sampling points: Slovakia A (SVK A); Germany (DEU); Poland A (POL A); Poland B (POL B); Slovakia B (SVK B); Slovakia C (SVK C); Romania A (ROU A); Romania B (ROU B); Bosnia A (BIH A); Bosnia B (BIH B); Italy (ITA); Bulgaria (BGR); and Spain (ESP). A standardized rank (1 for the highest rank value; 26 for the lowest one) was assigned to each result (gram-positive and gram-negative bacteria, fungi, total nitrogen and C_{org} contents, soil moisture, total microbial number, pH, PDA, ADA, CA, and UA, and total and organic P content). The total rank (red colour in the table for low rank value; green for high rank value) of each sample was calculated as the sum of each parameter rank. UP, ranking for 0–15 cm soil sample results; DOWN, 15–30 cm soil sample results. Overall: the results from both depths are considered together in the ranking calculation.

Table 4. Correlation (*r*) between sample coordinates (longitude, latitude, and elevation) and overall rank (see Table 3).

Samples	Latitude (N)	Longitude (E)	Elevation (m a.s.l.)	Overall rank (UP + DOWN)
Slovakia A	48.67797	19.47017	1180	238.5
Germany	49.08566	13.30653	1120	265.0
Romania A	45.49583	25.18778	1461	377.5
Romania B	45.53722	25.88382	1277	309.5
Slovakia B	49.17147	19.08182	767	315.0
Slovakia C	49.28517	16.73928	490	273.0
Poland A	49.62243	18.91460	520	352.5
Poland B	49.43298	20.90310	830	371.0
Bosnia A	43.72444	18.28583	1290	307.5
Bosnia B	44.64409	16.66843	524	306.0
Italy	46.11889	12.42972	1090	242.0
Bulgaria	42.67250	23.85083	1350	322.0
Spain	41.77556	2.456667	1186	181.5
Correlation *r*	**0.26**	**0.79**	**–0.1**	
p value	**0.19**	**0.0006**	**0.63**	

Note: The overall rank refers to the results from both depths (UP, 0–15 cm; DOWN, 15–30 cm of depth), and these are considered together in the ranking calculation.

displayed lower values, while the samples from Poland (POL A and B), Romania, and Bulgaria exhibited higher values. This could be explained by the geographical position of the sampling sites (Fig. 1), as most of the sites with lower values are located in Western Europe, while most of the sites with higher values are located in Eastern Europe. The correlation analysis (Table 4) illustrates how the longitude was strongly correlated with the overall rank analysis (r = 0.79; p = 0.0006), supporting the hypothesis that the differences observed could be due primarily to the geographical location of the sampling sites in terms of longitude.

PCA analysis

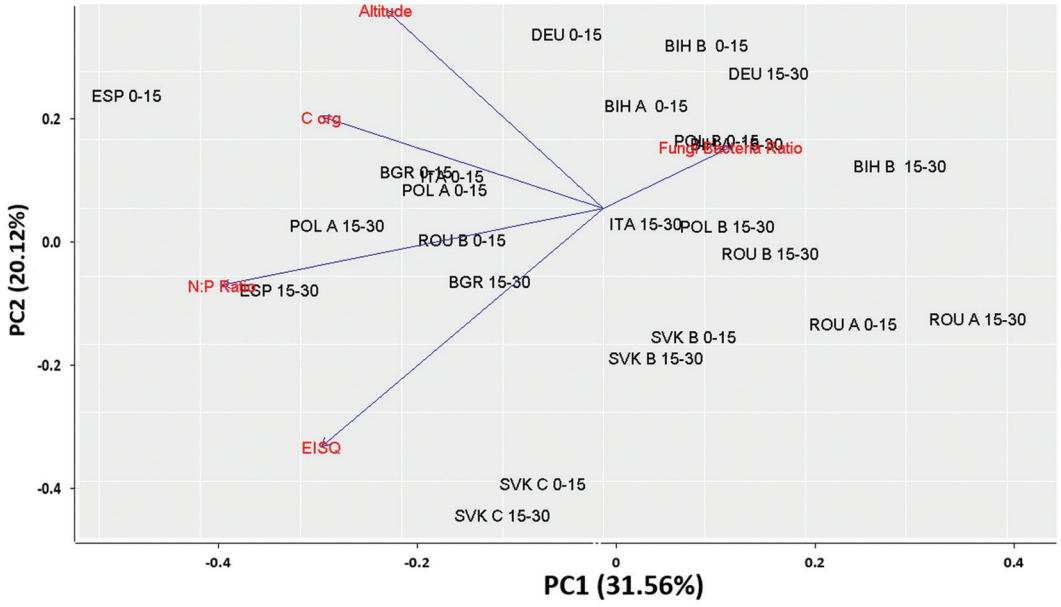
The sum of the PC1 and PC2 axes of the principal component analysis plot (Fig. 10) explains 51% of the variance among the sites. Samples from Spain (ESP), Bulgaria (BGR), Poland (POL A), and Romania (ROU B) are grouped in a cluster and characterized by high C_{org}, EISQ, N:P, and microbial abundance values. These parameters are strongly related because C_{org} influences both microbial activity and abundance (Nannipieri et al. 2002). In particular, the ESP 0–15 cm sample is distinct from the others due to its high C_{org} content as the main factor, but other factors (e.g., high EISQ value (Table 2) and high N:P value (Supplementary Table S3[2])) also influenced this result.

Samples from Germany (DEU), Bosnia (BIH A and B), and the Zadna Polana National Nature Reserve in Slovakia (SVK A) create another cluster characterized by high pH values and a low fungi–bacteria ratio. Finally, the other two Slovakian plots situated in the Low Fatra National Park Nation Nature Reserve (SVK B and C) and the two Romanian sites situated in the Southern Carpathians (ROU A and B) form another cluster characterized by low C_{org} and microbial abundance values and high EISQ values.

Discussion

Soil microbial communities provide several services in forests and have a fundamental role in terrestrial biogeochemical cycles; however, their spatial distribution and the variation in microbial activities have not been well researched (Crowther et al. 2019). Several biotic and abiotic factors affect the biogeographical

Fig. 10. Principal component analysis (PCA) of all data (with longitude, latitude, and elevation factors) for each sampling point (0–15 and 15–30 cm depth) considering the following factors: microbial abundance; pH; organic carbon (C_{org}); fungi–bacteria ratio; nitrogen–phosphorus ratio (N:P), and enzymatic indicator of soil quality (EISQ). The Eigenvectors (all the components) are highlighted in blue. Sampling plots: Slovakia A, B, and C (SVK A, SVK B, and SVK C, respectively); Germany (DEU); Romania A and B (ROU A and ROU B, respectively); Poland A and B (POL A and POL B, respectively); Bosnia A and B (BIH A and BIH B, respectively); Italy (ITA); Bulgaria (BGR); and Spain (ESP). [Colour online.]



distributions of soil microbial biomass, activity, and communities on geographical scales (Nannipieri et al. 2018).

The aim of this study was to find correlations between soil characteristics (biotic and abiotic factors) and latitude, longitude, or elevation. As already found by several authors, the effect of elevation on soil microbial communities is probably too complex to be indicated by a simple linear relationship, or it is obscured by local edaphic gradients and environmental conditions (e.g., moisture availability and soil evaporation rates) and site-specific factors (e.g., stand structure and species composition) (Adamczyk et al. 2019). In our samples, high enzymatic activities were found in the surface soil layer (0–15 cm), probably due to the high C_{org} content (Supplementary Table S3[2]). The N:P values indicate that the quantity of phosphorus in these soils is not limited.

Forest tree species have a significant effect on the soil microbiome by determining the quality of substrates (Bani et al. 2018). Beech litter is also easily decomposed due to its high content of water-soluble compounds (Priha and Smolander 1997), and this explains the high enzymatic activity that we found in the upper soil layer. Stand density and, consequently, forest management (Pioli et al. 2018) may also affect the amount of litterfall and, therefore, nutrient availability and microbial communities across soil layers. Changes in the dynamics of litterfall (and its components) reflect climatic patterns, namely those affecting water availability and nutrient supply (Khanna et al. 2009).

The enzymatic potential of the soils, which is an indicator of soil quality and offers an image of the intensity of soil activity (Bastida et al. 2008; Dick 2015; Schloter et al. 2018), varied within a wide range (0.6–0.9; Table 2), indicating that soils had a generally good enzymatic potential, from medium to high. These values reflect the microbial turnover and activity, which is widely known to be high in the rhizosphere (Koranda et al. 2011).

Although one composite sample was analyzed for each plot and for each sampling depth, the correlations in our study demonstrated that elevation was not the main factor determining the changes in abiotic or biotic characteristics at the sites investigated. Soil enzymatic activities and soil microbial communities were not affected by elevation, the effect of which was indirect and was presumably exerted predominantly through its influence on vegetation (presence of beech trees), as already found by several authors (Grayston and Rennenberg 2006; Koranda et al. 2011; Colin et al. 2017). Fungal dominance could be partially explained by vegetation types (Hackl et al. 2005; Zhang et al. 2013). Indeed, fungal structure and composition are strongly affected by tree species composition, and bacterial communities differ to a great degree between rhizosphere and bulk soils (Uroz et al. 2016; Nacke et al. 2016).

Although all the soils sampled were of the same pedological class (Cambisol), we found that the Spanish plot (in which temperature gradually increased while precipitation decreased over the last years, data not shown) had the highest values for C_{org}, EISQ, total N, and fungi and microbial numbers (Figs. 3 and 4; Table 2; Supplementary Table S3[2]) of any plot. This sampling site also had the lowest value obtained with the ranking procedure (Table 3, red colour) and differed particularly from the Romanian and Polish samples, which were among the most geographically distant plots (in longitude). Moreover, in Fig. 10, the Spanish plot (both sampling depths) is distinct from the other soil samples, with the highest C_{org} content, N:P values, and EIQS values. The Spanish beech plot is located in the Montseny Natural Park in Catalonia (northeastern Spain), which is the southwestern limit of the beech distribution area (Jump et al. 2006; del Río et al. 2018) and, as such, is warmer. In this marginal area for beech growth, the high C_{org} content may support microbial activity (Schloter et al. 2018) and, therefore, help trees to withstand stress conditions (Kumar and Verma 2018). Moreover, the amount of gram-positive bacteria (which are usually more predominant in soils of lower quality; Zhou et al. 2017) was among the highest found. As already found by other authors, abiotic factors (soil properties) influenced the microbial community along the

longitude gradient (Xue et al. 2018). Some exceptions that were found were probably due to abnormal precipitation in 2018, which may have influenced the microbial community at the time of sampling (Supplementary Table S1[2]). This trend could be due to the biogeography of soil biota or to a precipitation and drought gradient that we observed in the plots (Supplementary Table S1[2]). Biogeography of microorganisms has already been studied by various authors (King et al. 2010; Richter et al. 2018; Meyer et al. 2018), although there are no studies regarding microbial community geographical distribution in beech forests among different European countries, including those of climatic marginality (Mellert et al. 2016).

The correlation with elevation and ADA and, in general, the variation in the enzymatic potential of the soils pointed to an overall good enzymatic potential and an indication of the trajectory of soil quality (Dick and Kandeler 2005). Indeed, the level of enzymatic activity provides valuable information regarding the fertility status of soils (Nannipieri et al. 2002) and, in our case, of beech forest soils. The enzymatic potential is tied to substrate availability and soil pH and reflects microbial nutrient demand in the local edaphic and environmental settings.

In general, the microbial abundance in our study was lower in the deeper soil layer than in the upper soil layer. Unlike in the case of bacteria, the number of fungi (which is normally influenced by edaphic parameters or litter chemistry; Uroz et al. 2016) was generally higher in samples in the deeper soil layer than in the upper soil layer, as already found by other authors (Baumert et al. 2018). These differences were probably a result of changes in soil characteristics such as C_{org} or total N concentrations along soil profiles, reflecting litter chemical compositions. As an example, C-limited fungi could receive carbon through the mycelium connected to the root apparatus of beech, which has deep roots (Baumert et al. 2018). Abundant mycorrhizal fungi, forming mutualistic relationships with the deep root apparatus of beech (Kariman et al. 2018), might also preferentially colonize the deeper soil layer.

The differences in rank analysis (Table 3) among the sampling sites in Spain, Slovakia, Germany, Italy, Poland, Romania, and Bulgaria could be explained by the geographic positions (Fig. 1). The majority of sites with lower rank values (in red in Table 3) are located in Western Europe, while most of the sites with higher rank values (in green in Table 3) are in Eastern Europe. The correlation analysis (Table 4) illustrates how longitude was strongly correlated with the overall rank analysis, supporting the hypothesis that the differences in rank number could be primarily due to the geographic location of the sampling sites in terms of longitude. This result was also confirmed by the PCA analyses, which highlighted that the Spanish plot is different from the others due to soil biotic and abiotic factors.

Conclusions

As forests provide valuable ecosystem services, it is important to study which soil biotic factors can support them. This study reports the differences in beech stands distributed at different longitudes, elevations, and latitudes. Overall, the elevation did not exert an important influence on enzymatic activities and soil microbial communities, and elevation and latitude did not significantly affect the soil parameters analyzed. The factor most influencing soil microbial communities was longitude; among the populations sampled, the one in Spain could be considered the most vulnerable to the effects of climate change (e.g., drought), being at the beech southern range edge, though displaying a high C_{org} content and N:P values, together with high soil quality. The results show that the analyses of structure (in terms of different microbial groups and their abundances) and functioning (e.g., activity) of the soil microbial community, if correlated to the chemical and physical properties of soil, are useful tools for developing and implementing indicators for beech forest stands in the climate-smart forestry context.

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