RESEARCH ARTICLE

Century-long recovery of mycorrhizal interactions in European beech forests after mining

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Abstract

Background and aims   Ecological restoration strategies are emerging globally to counteract biodiversity loss and ecosystem degradation. However, restored ecosystems may not reach undisturbed biodiversity and functionality. One reason of this limited success may be a focus on short-term recovery of diversity, composition, or isolated functions. These simplified metrics may underestimate the real time ecosystems need to recover. Thus, studies of more complex metrics, like biotic interactions, at larger timescales, are essential to understand ecosystem recovery.

Methods Using molecular identification, we assessed the recovery of the interactions between ectomycorrhizal (EcM) fungi and European beech (*Fagus sylvatica* L.) in two opencast iron mines in use since the fourteenth century and abandoned over 107 and 148 years.

Results Species richness, species diversity, *Basidiomycota/Ascomycota* abundance ratio and taxonomic distinctness of EcM fungi recovered to undisturbed values, whereas species composition was still different. Certain fungal functional traits (i.e. exploration and sporocarp types) also reached undisturbed values. Differences in soil pH and NH_4^+ affected the composition of the EcM communities associated with beech, suggesting that mining caused a long-term impact in soil biogeochemistry, that directly impacted beech-EcM interactions.

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Conclusion Mycorrhizal interactions require more than 150 years to recover following mining. Contrary to the rapid recovery response provided by simple metrics like species richness, recovery metrics with more ecological information, like the identity of plant-EcM interactions, may be still capturing signals of incomplete recovery.

Abbreviations

AIC_c	Corrected Akaike Information Criterion
ANOVA	Analysis of variance
EcM	Ectomycorrizal
NH_4^+	Soil ammonium
NO_3^-	Soil nitrate
PO_4^{2-}	Olsen phosphorus
PERMANOVA	Permutational multivariate analysis of variance
SOC	Soil organic carbon

Introduction

The accelerating pace of ecosystems' degradation compromises their ability to maintain the current levels of biodiversity, functions and ecosystem services (Newbold et al. 2015). In the last decades, ecological restoration has become a key tool to counteract this trend (Strassburg et al. 2020), as has been acknowledged with the declaration of 2021–2030 as the United Nations Decade of Ecosystem Restoration (UN Environment Assembly 2019). However, restored ecosystems may be less functional and diverse than those preserved (Moreno-Mateos et al. 2017). One of the reasons that might help explain this reduced success is that traditional restoration approaches are based on the recovery of simple attributes (e. g. endangered species population density or species diversity), or single functions (e. g. reduction of soil erosion) (Montoya et al. 2012; Moreno-Mateos et al. 2017). These simplified metrics are only measured for a few years after disturbance ends, ignoring that ecosystem recovery may take centuries (Curran et al. 2014; Rydgren et al. 2020).

In recent years, an increasing number of studies are emphasizing the need to focus on restoring biotic interactions, as they play a key role in the structural and functional recovery of ecosystems (Montoya et al. 2012; Moreno-Mateos et al. 2020). One of the most relevant interactions in forest ecosystems, especially in temperate and boreal ones, is the mutualistic interaction between woody plants and ectomycorrhizal (EcM) fungi (van der Heijden and Horton 2009). It is currently estimated that ca. 6000 plant species are involved in the association with ca. 20,000–25,000 EcM fungal species of *Basidiomycota* and *Ascomycota* (van der Heijden et al. 2015). These fungi form a mantle external to the root tissue enhancing plant water and nutrient access and stress tolerance (Tedersoo et al. 2010). Temperate and boreal forests are usually dominated by a few woody species that harbor rich and diverse EcM fungal communities (Buée et al. 2005). This high EcM species diversity commonly involves increased functional diversity, including traits related to the exploration of the surrounding soil based on the extent and pattern of hyphal development, so-called "exploration type" (e.g. short vs. long distance; Agerer 2001), or the type of sporocarp (e.g. hypogeous vs. epigeous). It has been widely recognized that the diversity of EcM fungal communities, as well as their structure and dynamics, are strongly influenced by climatic and edaphic factors, like soil pH and nutrient levels (Hawkins et al. 2015; de Witte et al. 2017).

Studies have shown that anthropic activities, such as mining or clearcutting, also affect forest EcM fungal communities, by reducing their species richness and diversity (Gebhardt et al. 2007; Sterkenburg et al. 2019), and changing their species composition (Glen et al. 2008; Walker et al. 2012) for many years after the end of the impact. However, like most studies on recovery from anthropogenic disturbance, they only cover a few decades. We need to improve our understanding of the recovery process in the long-term. This would help us estimate the recovery time required to reach similar structural and functional conditions to those existing before degradation started.

We studied the recovery of tree-EcM fungal interactions over the long term (> 100 years) after mining impacts. We compared the EcM fungi associated with European beech (*Fagus sylvatica* L.) in two opencast iron mines in use since the fourteenth

century and abandoned for more than 100 years with the preserved surrounding forests. Both the mines and the surrounding forest have been protected since 1919 and no major impacts have happened in them since then. We sampled EcM fungal tips present in tree roots and characterized beech EcM fungal communities by using metrics with distinct levels of ecological information (i.e. species richness, species diversity, taxonomic distinctness, *Basidiomycota* vs. *Ascomycota* relative abundances, and species composition). Alpha diversity metrics were calculated for each studied site while community dissimilarity between sites (beta diversity) was calculated using Bray-Curtis index. Specifically, we aimed to answer three questions. 1) Are there differences in the level of recovery reached by the metrics selected to characterize beech EcM fungal communities? We hypothesize that the more ecological information included in the metric, the lower the level of recovery and the longer the time to reach pre-disturbance values. These predictions are based on findings of longer recovery times for species composition than for species richness, as the former incorporates information about species identity (Curran et al. 2014; Rodríguez-Uña et al. 2019; Rydgren et al. 2020). Expanding our preliminary low-data intensive results showing a recovery of EcM fungal species richness but not of species composition (with presence/absence; Rodríguez-Uña et al. 2019), this study includes a significantly larger amount of data of species diversity, species composition (with abundances), taxonomic distinctness, and phylum. 2) Are there fungal functional traits (i.e. exploration type and sporocarp type) associated with disturbed or preserved forests? Previous studies reported a higher prevalence of short-distance exploration types in older forests. They argued that carbon fixation by trees might decrease with stand age, hindering the EcM fungal production of abundant and long hyphae, which are carbon costly tissues. (Rudawska et al. 2018; Wasyliw and Karst 2020). Therefore, we hypothesize that long-distance exploration type will be scarcer outside the mines. We also hypothesize that sporocarp structures associated with long-distance dispersal will be more abundant inside than outside the mines. The reason for that is that they would have better chances to reach the recovering forest than sporocarps, which are associated with a short-distance dispersal strategy (e.g. hypogeous gasteroid) and whose chance to reach the new forest

are low (Kranabetter and Friesen 2002; Halbwachs et al. 2016). 3) Is the legacy of mining on soil biogeochemistry affecting the recovery of beech EcM fungal communities? Considering the effects of opencast iron mining on soil biogeochemistry (e.g. soil extirpation, severe pH changes, and in most cases the elimination of soil nutrients) and, subsequently, on soil microbiota (Deng et al. 2020), we hypothesize that the legacy of centuries of mining, is still affecting the composition and function of soil microbial communities.

Methods

Study area

The study area is located in the European beech forest of Artikutza (3638 ha), northern Spain (43°10′56.6″N 1°47′41.2″W; Fig. 1). This area has a temperate oceanic climate, with a mean annual temperature of 12 °C (19 and 6 °C in the hottest and coldest months respectively) and an annual rainfall of 2500 mm (Peralta et al. 2018). The soils are mainly Cambisols created from metamorphic materials, with base-metal veins, mainly iron (Galán et al. 2014). These iron deposits were exploited at least from the fourteenth century until 1919, when Artikutza was acquired by the San Sebastián City Council. Since then, no human uses (except recreational activities) were permitted, allowing the forest to recover. The dominant vegetation association is the Atlantic acidophilic beech forest (*Saxifrago hirsutae-Fagetum sylvaticae*; Peralta et al. 2018).

To study the recovery of the EcM fungal interactions with European beech trees, we selected two abandoned opencast iron mines. The mines are located in opposite sides of the same hill, whose altitude range from 250 to 750 m a. s. l. Mine 1, oriented to the SE, covers 7.7 ha (Fig. S1a). Mine 2, oriented to the NW, is divided into three cuts (i.e. inverted cone shape exploitations that evolve in depth, typical from opencast metal mines) and covers 4.3 ha. We used several dating approaches to estimate the moment when mining activities ceased in each cut (see Supplementary text). The preserved surrounding forests historically suffered minor impacts, mainly pollarding for charcoal production, until 1919 (Fig. S1b).

Sampling and processing

We found three main limitations to select trees inside the mines. First, the varying and steep slopes (up to 64%) could affect EcM associations with trees (Zhang et al. 2013). To prevent this, we selected trees growing in slopes below 25%. Second, to cover the whole time period since the end of mining, we selected all the trees without decaying evidence in each mine with a diameter at breast height of at least 40 cm. These criteria allowed us to include nine trees inside mine 1 and eight inside mine 2 (Fig. 1). We also sampled all trees at a distance between 10 m and 100 m from the edge of the mine with a diameter of at least 40 cm in the preserved forest, avoiding trees near to old ore extraction roads. These criteria allowed us to include six trees outside the mines.

We collected fine roots from each tree in spring and autumn of 2017. In each season, we collected 500 ml of roots from four plots 25×25 cm^2 and 10 cm deep, given that their abundance deeply decreases below this depth (Neville et al. 2002). The plots were 1.5 m away from the tree and at 90° from each other. Both the interior and the preserved surroundings of the mines are characterized by a strong beech dominance, with virtually no risk of collecting roots of other EcM tree species (see Fig. 1). We rotated the

collection points 45° to the right in autumn to avoid collecting roots from the same plot. We pooled the roots from the four plots of each tree (total: 18, 16 and 12 root samples from mine 1, mine 2 and outside, respectively), for each season. To preserve the roots, we kept them with their own soil in plastic bags at 4 °C, until they were processed within the following 48 hours. From each sample, we randomly selected five 5-cm root segments. Using stereo-microscopes, we identified the EcM morphotypes following Agerer (1987-2012) and Agerer (2001) and quantified the number of root tips of each morphotype per segment. To maximize the resolution of EcM identification, at least one tip of each morphotype distinguished in each segment from each sample was preserved in hexa-decyl-trimethyl-ammonium bromide (CTAB) for later DNA extraction and sequencing (total: 691 root tips).

We also collected 100 g of soil at each sampled tree to estimate the soil carbon and nutrient concentration. Soil samples were dried at 40 °C for 48 hours and homogenized. Soil samples were then analyzed using spectrophotometry to estimate the concentration of ammonium (NH_4^+), nitrate (NO_3^-) and Olsen phosphorus (PO_4^{2-}) (Olsen et al. 1954). The percentage of soil organic carbon (SOC), using the Walkley and Black (1934) method, and soil pH were also determined.

EcM identification and sequence bioinformatics

We sequenced the DNA of 691 EcM root tips following a modification of Murray and Thompson (1980) protocol. ITS1F-ITS4 primers were used for PCR amplification of the ITS rDNA region (White et al. 1990; Gardes and Bruns 1993). A semi-nested PCR (with ITS1F-ITS2 or ITS3-ITS4 primers) was needed for amplifying partial fragments in 14% of the sequences where the initial reaction failed. We repeated PCR reactions for 8% of the sequences with basidiomycete-specific primers ITS1F-ITS4B (Bellemain et al. 2010), when the first protocol did not render good quality reads to prevent the amplification of possible non-ECM ascomycete contaminants present in the root tips. When this reaction failed, a semi-nested PCR was carried out with ITS3-ITS4B primers. The amplification program consisted in a hot start at 95 °C for 5 min, followed by 35 cycles of 45,
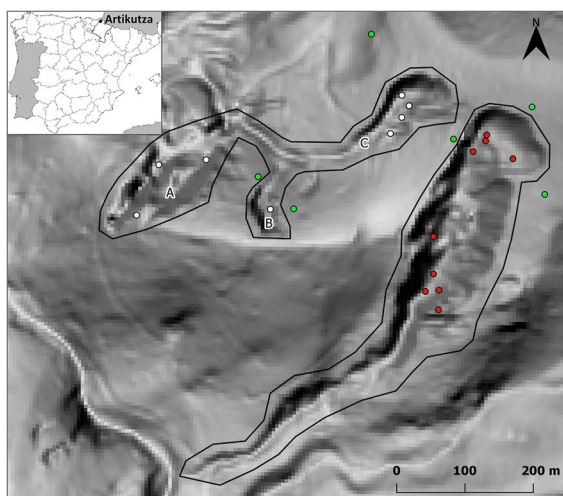

Fig. 1 Location of the study area and the sampled trees. Red dots indicate trees inside mine 1; white dots, trees inside cuts A, B and C of mine 2; and green dots, trees outside the mines

30 and 45 s at 94 °C, 54 °C and 72 °C, respectively; and a final elongation phase at 72 °C for 10 min. Results were checked in an agarose gel at 1%, and positive reactions were purified and sequenced with ITS4 (for whole ITS or ITS2 regions) or ITS1F (for ITS1 region). All sequences and chromatograms were checked to detect and correct reading mistakes.

We used Geneious Prime 2019.0.4 (https://www.geneious.com) to analyze 598 positive DNA sequencing results. Low quality ends were trimmed, prior to filtering by sequence quality and 165 sequences were discarded because of low quality. We clustered the 443 resultant sequences with a similarity threshold of ≥97% into operational taxonomic units (OTUs), using BLAST (https://www.ncbi.nlm.nih.gov/BLAST) to assign taxonomy (Anslan et al. 2016). We then discarded 17 sequences that matched non-EcM fungi and 18 sequences that could not be identified up to the genus or species level. When one sequence matched with two or more *species affinis* (i.e. closely related, but not identical species), we selected the species already identified in nearby beech forests. The insufficient resolution of the ITS region to identify the species inside certain genera may be one of the reasons why 57 sequences (12%) were not identified at the species level with ≥97% similarity. To reduce this proportion, they were aligned with the other sequences of their genus in Geneious. As genus *Tomentella* had the highest proportion (20 from a total of 27) of OTUs not affiliated to a taxonomically identified species, we ran a maximum likelihood phylogeny with one representative sequence of each OTU and their closest matching sequences at GenBank (see phylogenetic placement of sequences in Fig. S2), using MEGA X (Kumar et al. 2018). The final dataset included 408 DNA sequences, which were deposited in NCBI GenBank (accession numbers MW282331 - MW282739).

Statistical analysis

For subsequent statistical analysis, each sample was defined as the sum of the five root segments from each sampled tree in each season. Therefore, 18, 16 and 12 samples were analyzed from mine 1, mine 2 and outside, respectively, for each season. To answer question one (i.e. different recovery level of EcM fungal communities depending on the metric), we first examined the differences in alpha diversity metrics - species richness, species diversity (Shannon-Wiener index), and taxonomic distinctness - between the three locations. Species richness was computed as the number of different OTUs present in each sample. To calculate species diversity, species abundances were calculated as the total number of EcM root tips of each OTU counted in each sample. Taxonomic distinctness (Δ^+), is defined as the average path length through a taxonomic tree connecting all species pairs. This metric was calculated based on the taxonomic distance between every pair of species of each sample through a classification tree at six taxonomic levels (species, genus, family, order, class and phylum) (Clarke and Warwick 1998) (see dendrogram of taxonomic distances in Fig. S3). Species richness, species diversity and taxonomic distinctness were calculated using the package *vegan* (Oksanen et al. 2019) in *R* 3.6.3 (R Core Team 2021). Sampling effort was assessed by constructing species accumulation curves for the EcM fungal communities using *specaccum* function from *R* package *vegan*, with the "random" method (Fig. S4).

We fitted a linear mixed model for species diversity and taxonomic distinctness, with location (mine 1, mine 2 and outside) and sampling season (spring vs. autumn) as fixed effects and sampled tree as random effect, using the function *lmer* of *R* package *lme4* (Bates et al. 2015). For species richness, we used the function *glmer* of *R* package *lme4* to fit a generalized linear mixed model with the same fixed and random effects but choosing a Poisson error distribution. We compared the models based on the corrected Akaike Information Criterion (AIC_c; (Burnham and Anderson 2002)), selecting the most parsimonious one with a difference in AIC_c lower than two in comparison to the minimum AIC_c among all models. Model residuals were tested to meet model assumptions (Fig. S5a-c) and the species richness model was checked for overdispersion. We subsequently compared the mean relative abundances of each phylum (*Basidiomycota* vs. *Ascomycota*) between locations using an analysis of variance (ANOVA) with post-hoc Tukey pairwise tests. Data were logarithmically transformed to satisfy the criteria of normal distribution and homogeneity of variances.

Thirdly, we used beta diversity metrics to assess the effect of location and sampling season on beech EcM fungal communities. Community dissimilarity between the three sites was calculated using Bray-Curtis dissimilarity index (accounting for species

abundances) and tested with a permutational multivariate analysis of variance (PERMANOVA). We used the *adonis* function of the *R* package *vegan*, with 9999 permutations. To visualize the similarity of EcM fungal communities between the trees in mine 1, mine 2 and outside, we generated a non-metric multidimensional scaling plot (NMDS) using the Bray-Curtis similarity index in the *metaMDS* function of the *R* package *vegan*. Finally, we identified those species (or combinations of them) associated with mine 1, mine 2 or outside using the indicator species analysis (Dufrêne and Legendre 1997) from the *multipatt* function in the *R* package *indicspecies* (De Cáceres and Legendre 2009), with 9999 permutations. This analysis assesses the relationship between species abundance and groups of sites, and the statistical significance of such associations.

Following question two (i.e. certain EcM functional traits associated with disturbed/preserved forest), we compared the mean relative abundances of each exploration type (contact-short, medium, or long) and sporocarp type (corticioid, gasteroid, pezizoid, stipitate, or without sporocarp) between locations using an ANOVA with post-hoc Tukey pairwise tests. The gasteroid reproductive structure data were log-transformed to meet a normal distribution and homogeneity of variances. Assignments of exploration type were based on Agerer (2001, 2006), Ostonen et al. (2017) and Defrenne et al. (2019), and assignments of sporocarp type were based on Agerer (2001) and the *FungalTraits* database (Põlme et al. 2021).

To assess question three (i.e., mining legacy on soil biogeochemistry still affecting the recovery of EcM fungal communities), we tested the influence of location in each soil variable (SOC, NH_4^+, NO_3^-, PO_4^{2-}, and pH) using an ANOVA with post-hoc Tukey pairwise tests. NH_4^+, PO_4^{2-} and pH data were log-transformed to satisfy normal distribution and homogeneity of variances. We determined whether these soil variables were correlated to each other by estimating the pairwise Pearson's regression coefficient. Secondly, we fitted linear regression models including all soil variables to test their effect on species richness, species diversity and taxonomic distinctness. Thirdly, we ran a PERMANOVA analysis for the effect of all soil variables (SOC, NH_4^+, NO_3^-, PO_4^{2-}, and pH) on beech EcM fungal communities. Finally, a canonical correspondence analysis (CCA) was conducted to visualize the ordination of beech EcM fungal communities of mine 1, mine 2 and outside in relation to the soil variables.

Results

Does different metrics reach distinct recovery levels for EcM fungal communities?

The obtained 408 sequences corresponded to 120 EcM fungal OTUs (hereafter referred to as "species"), 99 (83%) *Basidiomycota* and 21 (18%) *Ascomycota*, within 38 genera across the 23 sampled trees inside and outside the mines. The most abundant genera were *Tomentella*, *Cenococcum*, *Entoloma* (only present in the mines), *Lactarius*, *Inocybe*, *Russula,* and *Sebacina* (Fig. S6), which have also been identified in nearby beech forests (Goicoechea et al. 2009; Sarrionandia et al. 2009). The number of unique species detected out of the total number of species in each location was 21 out of 56 in mine 1, 25 out of 53 in mine 2, and 29 out of 54 outside. Mine 1 and mine 2 share eight species, mine 1 and outside share 10 species, mine 2 and outside share three species and the three locations share four species (Table S1).

Cenococcum geophilum was the most common species in all locations, with a relative abundance of $7.88\% \pm 5.04\%$, $7.85\% \pm 3.86\%$ and $24.18\% \pm 7.47\%$ (mean $\pm$ s.e.) in mine 1, mine 2 and outside, respectively. Other abundant species but only present outside the mines were *Sistotrema* sp.1, *Lactarius blennius,* and *Russula fellea*. The most abundant species appearing inside both mines but not outside were *Entoloma bryorum*, *Laccaria laccata, Inocybe maculata* and *Cyanoboletus pulvurulentus*. *E. bryorum* was identified as the indicator species of mine 2 ($P = 0.002$; Fig. 2; Table S2).

Mine abandonment dating results suggest that mine 1 and cut C of mine 2 were abandoned between 125 and 148 years before sampling, and cuts A and B in mine 2 were abandoned 107 years before sampling (see Supplementary text). The species richness per tree was 5.50 ± 1.76, 6.13 ± 2.53 and 6.18 ± 2.61 (mean $\pm$ s.e.) in mine 1, mine 2 and outside, respectively. The species diversity per tree was 1.33 ± 0.34,
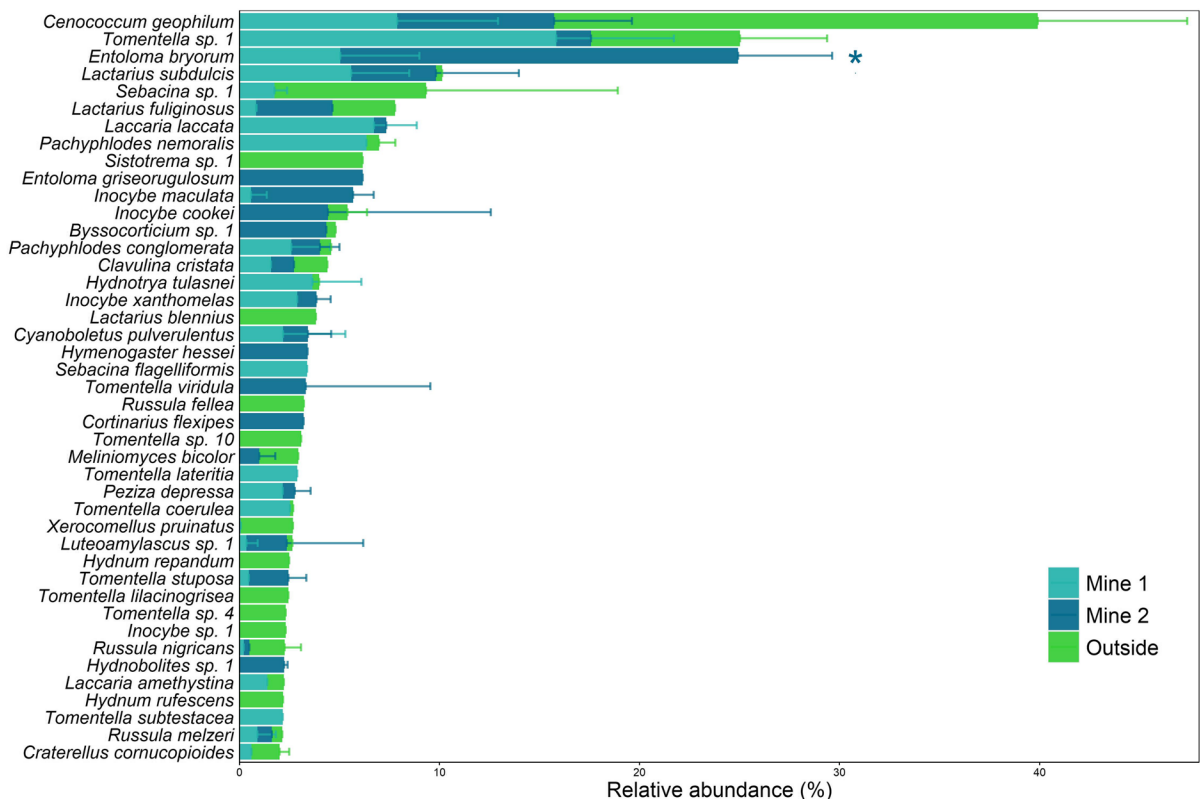
Fig. 2 Relative abundance of ectomycorrhizal fungal species found in European beech roots inside and outside the mines. It only shows species with >2% total relative abundance. Bars indicate means and standard error. Asterisk indicate the best indicator species of mine 2 as determined by the indicator species analysis

1.43 ± 0.27 and 1.41 ± 0.41 (mean $\pm$ s.e.) in mine 1, mine 2 and outside, respectively. The taxonomic distinctness per tree was 60.26 ± 10.18, 64.48 ± 8.67 and 63.95 ± 8.76 (mean $\pm$ s.e.) in mine 1, mine 2 and outside, respectively. We found no differences in species richness, species diversity and taxonomic distinctness between the three sampled locations or the sampling season, i.e. the model that excluded the effect of location and season had the minimum AIC_c (Table S3). The PERMANOVA model revealed a significant effect of the location on beech EcM fungal community composition, but not of the sampling season (Table 1). NMDS visualization of beech EcM fungal communities was consistent with the effect of location (Fig. 3). The relative abundance ratio of *Basidiomycota/Ascomycota* per tree was 0.75 ± 0.07, 0.73 ± 0.07 and 0.81 ± 0.05 (mean $\pm$ s.e.) in mine 1, mine 2 and outside, respectively, with no significant differences between them (Fig. S7a).

Are there fungal functional traits associated with disturbed or preserved forests?

When EcM fungal species were grouped by sporocarp type, we found a higher mean relative abundance of the "stipitate" group in mine 2 with respect to mine 1 and outside (ANOVA: $F_{2,20} = 11.40$, $P < 0.05$; followed by post-hoc Tukey pair-wise tests; Fig. S7c). However, we found no differences between locations when grouped according to their exploration type (Fig. S7b).

Does the legacy of mining on soil biogeochemistry affect the recovery of EcM fungal communities? Soils from the three sampled locations had

Table 1 Results of the PERMANOVA analysis to test the effect of location (mine 1, mine 2 and outside the mines) and sampling season (spring and autumn), and soil variables on ectomycorrhizal fungal community composition found in European beech roots

Location & sampling season					
Source	Df	Sum Sq	Pseudo-F	R^2	p(perm)
Location	2	2.066	2.482	0.103	**<0.001**
Sampling season	1	0.466	1.121	0.023	0.292
Residuals	42	17.484		0.873	
Total	45	20.018		1.000	
Soil variables					
Source	Df	Sum Sq	Pseudo-F	R^2	p(perm)
pH	1	0.832	2.013	0.087	**0.001**
NH_4^+	1	0.525	1.271	0.055	0.121
NO_3^-	1	0.341	0.826	0.036	0.786
SOC	1	0.427	1.034	0.045	0.409
PO_4^{2-}	1	0.446	1.080	0.047	0.328
Residuals	17	7.023		0.732	
Total	22	9.595		1.000	

Df degrees of freedom, *Sum Sq* sum of squares, *Pseudo-F F* value by permutation, *p*(perm): *p*-values based on 9999 permutations (statistically significant *p*-values are shown in bold)

different NH_4^+ (ANOVA: $F_{2,20} = 11.40$, $P < 0.001$) and PO_4^{2-} (ANOVA: $F_{2,20} = 3.78$, $P = 0.04$) concentrations and pH ($F_{2,20} = 11.39$, $P < 0.001$) values (Fig. 4). However, SOC and NO_3^- concentrations were not different among the three locations (Fig. 4; Table S4). Post-hoc Tukey pairwise tests detected a higher pH and lower NH_4^+ in mine 2 with respect to mine 1 (pH: $P = 0.005$, NH_4^+: $P < 0.001$) and outside (pH: $P < 0.001$, NH_4^+: $P < 0.001$). In contrast, PO_4^{2-} was higher in mine 1 compared to mine 2 ($P = 0.02$) and to outside ($P = 0.03$). NH_4^+ and pH were negatively correlated ($\rho = -0.46$, $P = 0.03$; Fig. S8). We found no effects of soil variables on species richness, species diversity, or taxonomic distinctness (Table S5).

The PERMANOVA model indicated that pH but not SOC, NH_4^+, NO_3^- and PO_4^{2-} influenced beech EcM fungal communities (Table 1). CCA visualization shows a separation of beech EcM fungal communities of mine 2 from mine 1 and outside, mainly explained by the effect of pH, followed by an effect of NH_4^+ (Fig. 5; Table S6).

Discussion

Our results confirm hypothesis one, which states that metrics including more ecological information require longer recovery times. The linear mixed models and the ANOVA analysis revealed that, after more than 148 and 107 years of opencast mining abandonment in mine 1 and mine 2, respectively, species richness, species diversity taxonomic distinctness, and *Basidiomycota/Ascomycota* abundance ratio of EcM fungi associated with beech trees have recovered to values present in the preserved surrounding forest. However, the PERMANOVA analysis on the Bray-Curtis dissimilarity matrix showed that EcM fungal species composition was still different in former mines compared to sampled non-mined areas. These results are in line with a preliminary study showing no differences inside and outside the mines for the EcM fungal species richness, but reporting differences in their species composition when presence/absence data was considered (Rodríguez-Uña et al. 2019). In this new study, we broaden this approach by reporting a recovery of the EcM fungal species diversity, species composition with abundance data, taxonomic distinctness, and phylum. Other studies found similar outcomes for EcM fungi associated with *Quercus rubra* in North America which recovered up to 63% of their species richness after 43 years since mining cessation (Gebhardt et al. 2007). EcM fungal species richness in jarrah forest in Australia recovered in 16-year-old rehabilitated mine sites, but species composition did not (Glen et al. 2008). A meta-analysis from temperate, Mediterranean and boreal planted or secondary forests estimated that EcM fungal species richness required on average 90 years (between 45 years to unrecoverable at 95% prediction limits) to reach old-growth forests' values (Spake et al. 2015). More broadly, a global meta-analysis reported that species richness in restored forests may converge to reference levels within a century, while the recovery of species composition may take up to an order of magnitude longer (hundreds to thousands of years) (Curran et al. 2014).

Although both mines share EcM fungal species with the preserved surroundings, some species that are abundant either inside or outside the mines are absent in the counterpart location. These dissimilarities may be driven by different abiotic conditions and
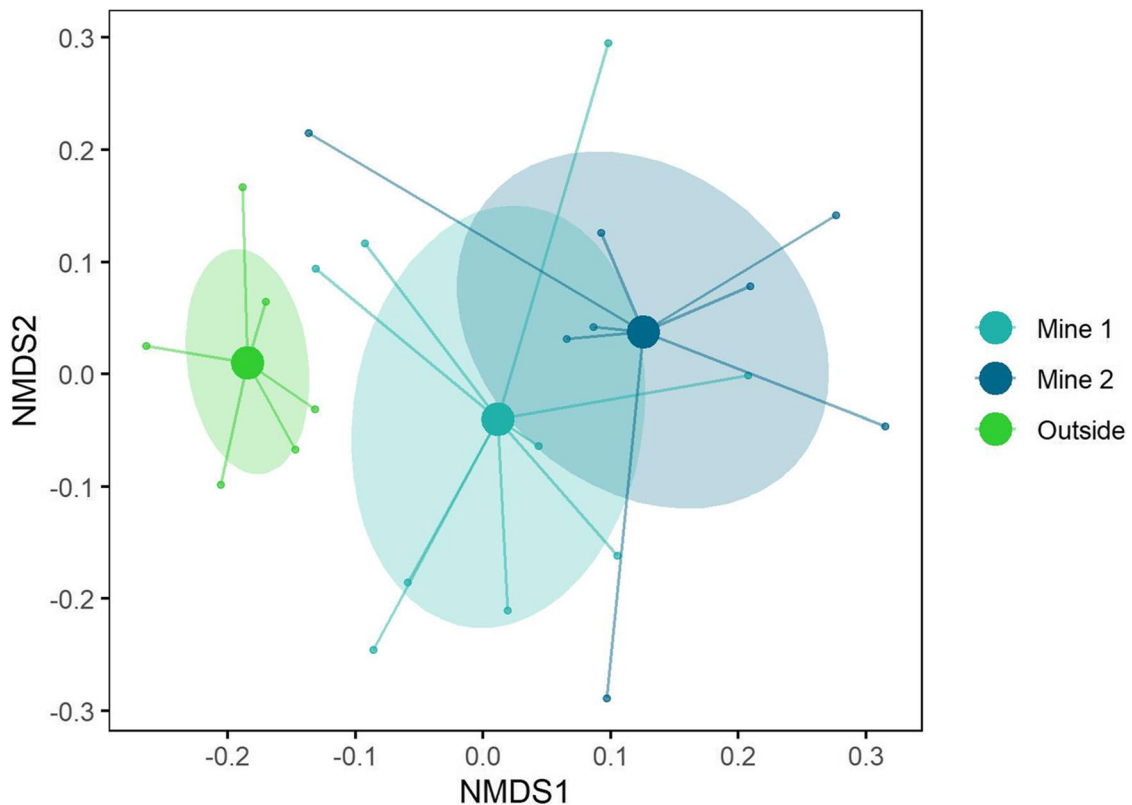
Fig. 3 Non-metric multidimensional scaling (NMDS) plot of the ectomycorrhizal fungal communities found in European beech roots inside and outside the mines (stress value = 0.19). Points on the ordination space represent sampled trees based on Bray-Curtis dissimilarity indices. Centroids and standard deviation ellipses of the three locations are also overlaid

distinct forest-recovery stages (Smith et al. 2002; van der Linde et al. 2018). For example, *L. laccata*, known for its ability to assimilate NO_3^- (Finlay and Frostegård Åand Sonnerfeldt 1992; Hobbie et al. 2008), was only present inside the mines, mainly in mine 1, where we found the highest concentration of NO_3^-. Although it would have been desirable to increase the sampling effort outside the mines, we still detected that its EcM fungal communities are characterized by *i)* the dominance of *C. geophilum*, a generalist in temperate forests (Douhan and Rizzo 2005; Gebhardt et al. 2007); *ii)* the presence of acidophilic species with a preference for long-established beech forests, like *Lactarius blennius* (Sarrionandia et al. 2009; Correia et al. 2021); and *iii)* an increasing abundance of species of the genus *Russula*, commonly found in mature forests (Durall et al. 2006; Twieg et al. 2007).

These results might indicate that EcM fungal communities inside the former mines have not yet recovered to reference more mature stages. Other studies based on temperate and boreal forests also demonstrated an evolution towards old-growth EcM fungal communities during secondary forest succession (Twieg et al. 2007; Spake et al. 2016).

The higher similarity of beech EcM fungal communities between mines compared to the surrounding preserved forest, together with the effect of location (i.e. mine 1, mine 2 or outside the mines) on those interactions, suggests that previous mining impacts explain part of the differences in species composition. To test the influence of previous mining impacts, it would have been preferable to include replicate sites from other mined forests. Unfortunately, the uniqueness of the study area in the densely populated
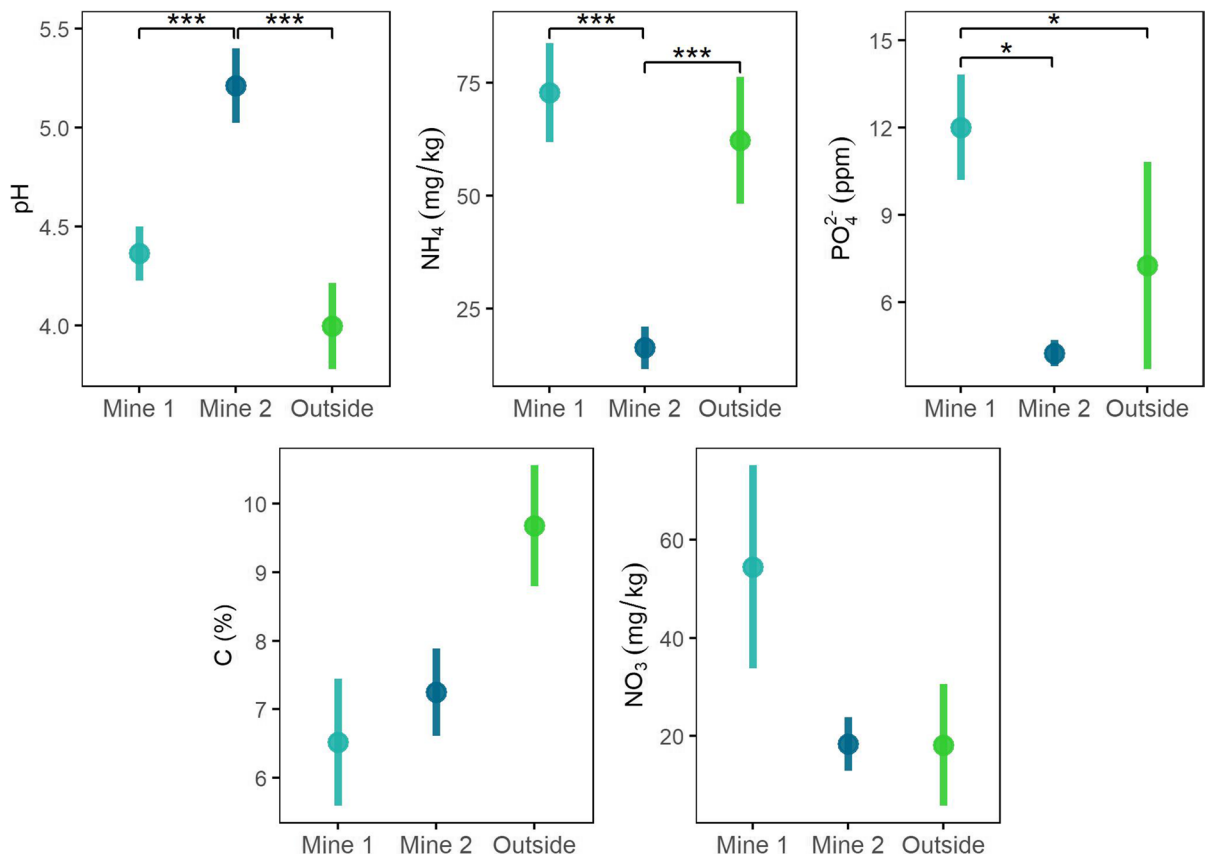
Fig. 4 Mean value (± standard error) of the soil variables measured (soil organic carbon (SOC), ammonium (NH_4^+), nitrate (NO_3^-), Olsen phosphorus (PO_4^{2-}-P) and pH) inside and outside the mines. Asterisks indicate significant differences (*: $P < 0.05$, ***: $P < 0.001$) obtained from Tukey post-hoc pairwise comparisons of the ANOVA results (see Table S4)

temperate forests in Western Europe makes it ideal for studying long-term recovery but hampers the possibility to find other sites with similar environmental conditions and resource exploitation approaches. The study sites were affected by the same impact and for the same period of time, recovering for more than 100 years. Nevertheless, the dissimilarity between EcM fungal communities from disturbed and preserved forests was higher for mine 2 than for mine 1. We propose two ways to explain this. First, the difference in mining ending dates between mines, as activities ended later in cut A and B of mine 2 (in 1910) compared to cut C (in 1892 ± 15 years) and mine 1 (in 1879 ± 25 years). A second explanation may be the difference in soil chemistry, as suggested by our question three. Our third hypothesis about the effects of

legacy of mining on soil microbial communities was confirmed by the ANOVA. We found different values of pH and NH_4^+ in mine 2 compared to mine 1 and outside the mines. The CCA and PERMANOVA analyses evidence that the values of pH and NH_4^+ were the strongest determinants of beech EcM fungal communities in our study area. These findings parallel large-scale experimental studies in beech forests revealing a major influence of pH and NH_4^+ on EcM fungal community composition (de Witte et al. 2017; van der Linde et al. 2018), but with no effect on species richness (Toljander et al. 2006; Geml et al. 2022).

Regarding our question two, the ANOVA confirmed our hypothesis that reproductive structures allowing a longer dispersion may have better chances to colonize recovering forests, as those EcM fungi
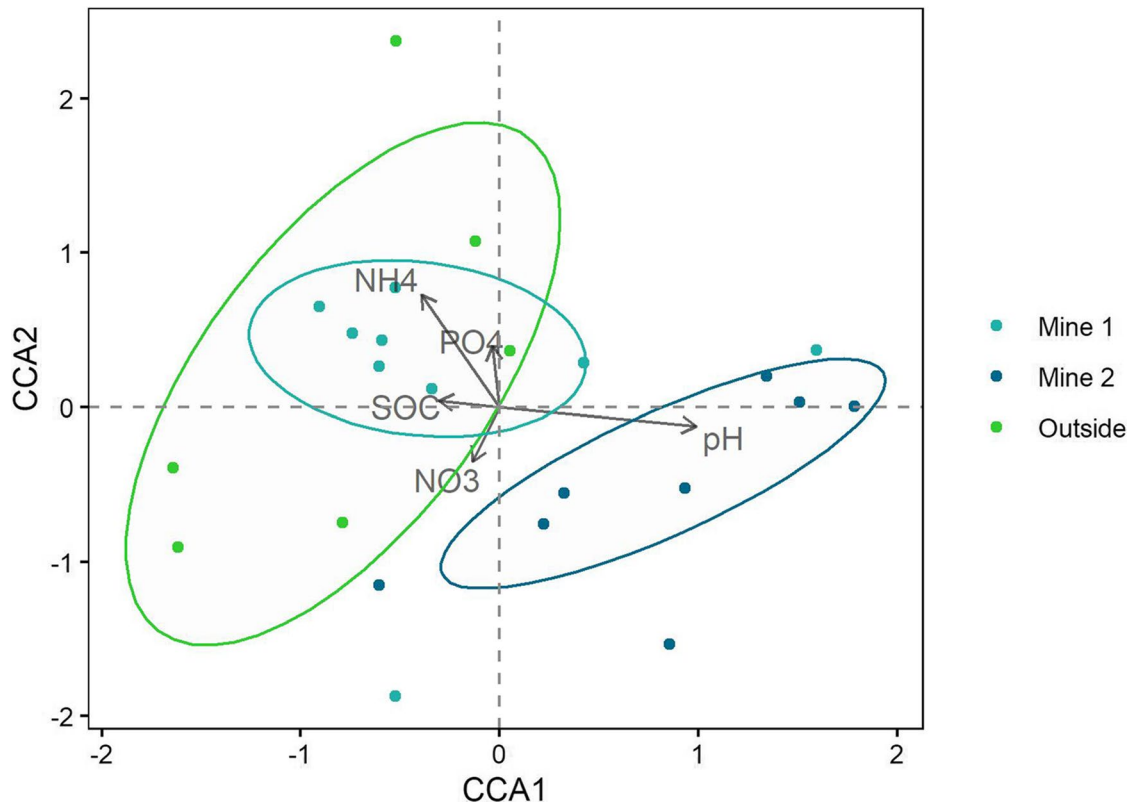
Fig. 5 Canonical correspondence analysis (CCA) plot of the ectomycorrhizal fungal communities found in European beech roots inside and outside mines. Points in the ordination space represent sampled trees based on the effect of the soil variables measured (soil organic carbon (SOC), ammonium (NH_4^+), nitrate (NO_3^-), Olsen phosphorus (PO_4^{2-}) and pH). Standard deviation ellipses of the three locations are also overlaid

with "stipitate" sporocarps were more abundant in mine 2, the youngest forest, than in the reference, older forest. Previous studies based on observed sporocarps instead of EcM sampled *in planta,* documented differences in the abundance of hypogeous and epigeous fungi among forest-recovery stages (Smith et al. 2002; Durall et al. 2006). In relation to the exploration type, the ANOVA showed no differences in their exploration type inside and outside the mines. This result contrasts with our initial hypothesis that long-distance exploration type will be scarcer outside the mines and other studies highlighting differences in the exploration type among forest successional stages (LeDuc et al. 2013; Rudawska et al. 2018; Wasyliw and Karst 2020). Although this fungal functional trait is considered an adequate indicator of restoration success in later stages (Avis et al. 2017),

the oldest stand sampled in these previous studies was 87 years old, lacking insights about the dominant exploration type in older forests (> 100 years). Therefore, our findings suggest that the effect of mining on the exploration type and sporocarp type recovered after more than 107–148 years of mine abandonment.

Overall, our outcomes suggest that while ecosystems may seem recovered over the long term based on metrics with low levels of ecological information, like those measuring the number and abundance of species, complete recovery may not be happening. We have found that alpha diversity recovered while species composition, i.e. the identity of beech associated EcM fungi species, did not recover after more than 107–148 years, stressing critical value of mature, preserved forests for fungal conservation (Kranabetter et al. 2018), as they

are also crucial natural inoculum reservoirs (Bauman et al. 2012). Metrics with more ecological information may better incorporate the assemblage of ecosystem complexity (Moreno-Mateos et al. 2020; Rydgren et al. 2020). Monitoring changes in these more complex metrics after restoration will likely provide a more accurate estimate of its performance. Restoration efforts could then favor EcM fungal communities existing in late-stages of forest recovery to accelerate this process. To improve this knowledge, further studies estimating how much nitrogen is absorbed by the plant thanks to each EcM fungal species (Pena et al. 2013) would enable to identify highly functional species or clusters of species to be used in forest restoration (Hawkins et al. 2015). In addition, assessing ecosystem recovery with metrics that incorporate more ecosystem complexity may also help to better predict the time required to full recovery and provide deeper insights into the real magnitude of ecosystem degradation.

Data availability The database created for this study will be deposited in Dryad.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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