RESEARCH ARTICLE

EcM fungal community structure, but not diversity, altered in a Pb-contaminated shooting range in a boreal coniferous forest site in Southern Finland

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Received 11 May 2010; revised 29 November 2010; accepted 12 December 2010.
Final version published online 20 January 2011.

DOI:10.1111/j.1574-6941.2010.01038.x

Editor: Philippe Lemanceau

Keywords
ectomycorrhizal fungi; lead (Pb) contamination; pyrosequencing; community analysis.

Abstract

Boreal forests contain diverse fungal communities that form essential ectomycorrhizal symbioses with trees. To determine the effects of lead (Pb) contamination on ectomycorrhizal fungal communities associated with the dominant pine (*Pinus sylvestris* L.), we surveyed sporocarps for 3 years, analyzed morphotyped ectomycorrhizal root tips by direct sequencing, and 454-sequenced fungal communities that grew into in-growth bags during a 2-year incubation at a shooting range where sectors vary in the Pb load. We recorded a total of 32 ectomycorrhizal fungi that formed conspicuous sporocarps, 27 ectomycorrhizal fungal phylotypes from 294 root tips, and 116 ectomycorrhizal fungal operation taxonomic unit (OTUs) from a total of 8194 internal transcribed spacer-2 454 sequences. Our ordination analyses by nonparametric multidimensional scaling (NMS) indicated that the Pb enrichment induced a shift in the ectomycorrhizal community composition. This was visible as indicative trends in the sporocarp and root tip data sets, but was explicitly clear in the communities observed in the in-growth bags. The compositional shift in the ectomycorrhizal community was mainly attributable to an increase in the frequencies of OTUs assigned to genus *Thelephora* and to a decrease in the OTUs assigned to *Pseudotomentella*, *Suillus*, and *Tylospora* in Pb-contaminated areas when compared with the control. While the compositional shifts are clear, their functional consequences for the dominant trees or soil ecosystem function remain undetermined.

Introduction

Communities of ectomycorrhizal fungi are highly diverse and variable in space (Taylor *et al.*, 2000) and time (Koide *et al.*, 2007; Walker *et al.*, 2008; Jumpponen *et al.*, 2010). Hundreds – if not thousands – of ectomycorrhizal species can be found in the boreal climatic region (Dahlberg, 2002; Knudsen & Vesterholt, 2008). Typically, several ectomycorrhizal species inhabit roots of an individual tree (Saari *et al.*, 2005) and practically all fine roots of coniferous trees are colonized by ectomycorrhizal fungi (Taylor *et al.*, 2000). Because of the temporal and spatial heterogeneity of the ectomycorrhizal communities, studying their structure and dynamics is a challenging task. This is especially true for species that fruit infrequently or produce inconspicuous fruiting bodies. Fungi that produce epigeous sporocarps more or less regularly are relatively well known, but the others, including those that form hypogeous or resupinate sporocarps, remain poorly known (Köljalg *et al.*, 2000; Horton & Bruns, 2001).

The mycorrhizal fungi are fundamental in ecosystem functioning. These fungi are responsible for carbon sequestration and for the majority of the nitrogen and phosphorus cycle in soil (van der Heijden *et al.*, 2008). Thus, environmental stresses that may have an impact on ectomycorrhizal fungi may also more broadly impact the functioning of the entire ecosystem. Lead (Pb) is considered highly toxic: Pb contamination in soil has been reported to decrease microbial biomass and alter the microbial community structure (Kuperman & Carreiro, 1997; Rantalainen *et al.*, 2006).

Anthropogenic activities like recreational shooting at dedicated shooting ranges, or military artillery shelling areas, may lead to substantial Pb enrichment. To exemplify, the annual Pb discharge on a recreational shooting range can range from 120 to 15 000 kg in a year depending on the shooting activity (Sorvari *et al.*, 2006). On a local scale, shooting ranges may thus constitute a threat to various organisms (Stansley *et al.*, 1997; Vyas *et al.*, 2000; Lewis *et al.*, 2001). The effects of Pb on ectomycorrhizal fungi are not well known, but in general, wide interspecific and intraspecific variations in metal tolerance of ectomycorrhizal fungi may exist (Hartley *et al.*, 1997).

The Hälvälä shooting range in southern Finland, the focus of our previous (Salminen *et al.*, 2002; Rantalainen *et al.*, 2006; Hui *et al.*, 2009) and current studies, contains up to 50 g Pb kg^{-1} of dry soil (Rantalainen *et al.*, 2006). Although most of this Pb may remain in the pellets for extended periods of time (Jørgensen & Willems, 1987), it is continuously physically and chemically mineralized into soluble forms that have the potential to bioaccumulate and have adverse biological effects (Labare *et al.*, 2004; Hartikainen & Kerko, 2009; Hui *et al.*, 2009). In our ecosystem-scale research in Hälvälä, phospholipid fatty acid analyses indicate reduced microbial biomass and suggest qualitative changes in the microbial communities as a result of Pb contamination. It is also possible that the Pb contamination acts as a contemporary evolutionary force selecting for more Pb-tolerant soil microbial communities in the contaminated soils in comparison with the microbial communities in the control soils that have remained free of Pb contamination (Rantalainen *et al.*, 2006; Hui *et al.*, 2009). Indeed, Colpaert *et al.* (2000) have suggested that selection pressure due to heavy metal contamination may cause genetic drift and reduce genetic variation in ectomycorrhizal populations.

The goal of the present study was to target the communities of ectomycorrhizal fungi and their responses to long-term Pb contamination. It is notable that the Pb contamination has been accumulating since 1964 (Turpeinen *et al.*, 2000) – for more than four decades at the time of this study. To meet the general goal of this study, we surveyed sporocarps of the ectomycorrhizal fungi for 2 years, analyzed the ectomycorrhizal communities by direct sequencing of morphotyped root tips associated with the dominant pine (*Pinus sylvestris* L.), and 454-sequenced active fungal communities that grew into in-growth bags (Wallander *et al.*, 2001) during a 2-year incubation. We hypothesized that at least some of these important mutualists of pine are sensitive to Pb contamination and thus less common in the contaminated area, and that these sensitive fungi are replaced by more Pb-resistant species, which in part may explain the healthy appearance of the contaminated forest.

Materials and methods

Study site

The study site (Fig. 1) is a shot-gun shooting range located in a stand dominated by Scots pine (*P. sylvestris* L.) and a smaller component of spruce and birch trees in Hälvälä, southern Finland (61°00′N 25°80′E). The old shooting range was used from 1964 to 1987 and has been allowed to reforest naturally since 1987. At that time, the shooting sector was moved to an adjacent, new area, which is still presently in use. For our studies on the communities and functions of soil organisms as related to the soil Pb contamination, we divided the site in 2003 into three areas varying in their Pb contamination (Rantalainen *et al.*, 2006): (1) a noncontaminated control area (C), (2) a medium contaminated area within the old shooting range (M), and (3) a heavily contaminated area, also within the old shooting range (OC; referred to as a 'heavily contaminated area' in Rantalainen *et al.*, 2006). A fourth area at the new shooting range (NC) was included in the studies in 2005. The total dimensions of each of the areas are approximately $75 \times 75\ m^2$. Because of very uneven spread of the pellets within the shooting sectors (Hartikainen & Kerko, 2009), we chose randomly ten $1.5 \times 1.5\ m^2$ study plots within each of

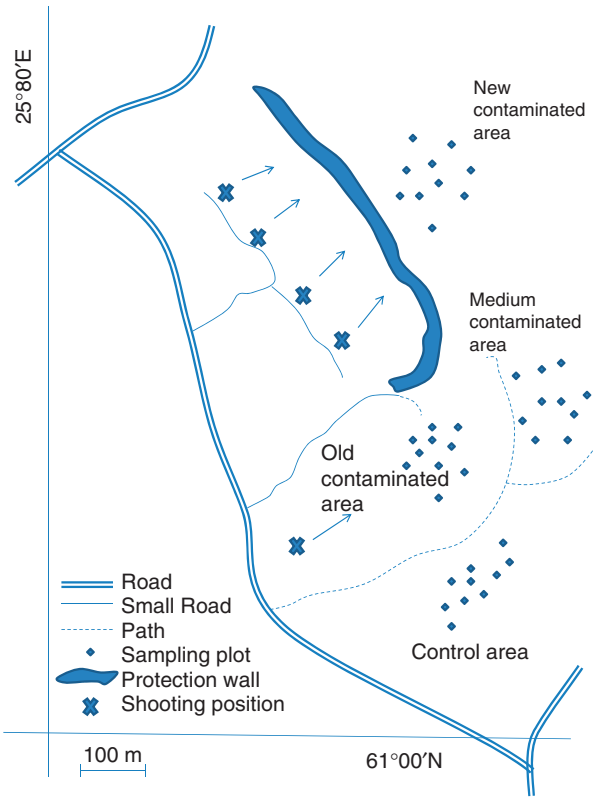

Fig. 1. Shooting range in Hälvälä showing the shooting sectors, areas, and approximate location of the plots.

Table 1. Environmental parameters in Hälvälä

Area	Total Pb concentration (mg kg^{-1} DW)*	Total Pb concentration (mg kg^{-1} DW)†	Water-soluble Pb concentration (mg kg^{-1} DW)†	pH*	Tree age*
C	75.6 ± 50^{a}	106 ± 88^{a}	3.2 ± 1.6^{a}	3.25 ± 0.13^{a}	21.6 ± 2.3^{a}
M	8700 ± 6485^{ab}	NA	NA	3.45 ± 0.22^{ab}	19.6 ± 2.3^{a}
OC	$18\,780 \pm 15\,770^{b}$	$42\,300 \pm 8634^{b}$	110 ± 35.5^{b}	3.54 ± 0.31^{b}	22.7 ± 1.9^{a}
NC	NA	$26\,700 \pm 8936^{ab}$	120 ± 19.6^{b}	NA	NA

Different letters in superscript indicate significant differences (Tukey's tests, $P < 0.05$) between study areas.
*Rantalainen *et al*. (2006). NC is not considered by this paper.
†Hui *et al*. (2009). M is not considered by this paper.
C, clean area; M, medium contaminated area; OC, old contaminated area; NC, new contaminated area; NA, not available.

the four areas at varying distances from each other (10–20 m from the nearest one; see the map in Rantalainen *et al*., 2006). The total Pb concentration (digestion in 8 M HNO_3) of the soil organic layer in each plot (Pb pellets excluded) was analyzed from C, M, and OC (Rantalainen *et al*., 2006) and from C, NC, and OC (Hui *et al*., 2009) (Table 1); data from those studies are used here as background information. Both measurements of Pb concentrations showed significant differences between the areas based on Tukey's HSD. The water-soluble Pb concentration showed a similar trend, as we observed a significantly higher soluble Pb concentration in NC and OC than in C, but the water-soluble Pb represented only 0.2–1% of the total Pb (Hui *et al*., 2009). Other site characteristics including ground vegetation were similar and the age of the dominant trees did not differ significantly (Rantalainen *et al*., 2006). Pb contamination appears to have significantly changed the soil chemistry by increasing soil pH by 0.2 (Rantalainen *et al*., 2006).

Sample collection

Sporocarps of epigeous ectomycorrhizal fungi were surveyed twice at the peak of the fruiting in the autumn in 2003 and 2004 and once in 2005 at the C, M, and OC areas; the NC area was added to these experiments after the sporocarp surveys had already been completed. The ectomycorrhizal (and unidentified potentially ectomycorrhizal) sporocarps were collected and stored dry for further identification. The species were first preliminarily identified based on morphology, and species potentially forming ectomycorrhiza with pine were separated from the rest. About two-thirds of the pine-associated species were common and distinguishable based on their morphological characteristics. From the remaining one-third, one specimen per species was chosen for sequencing, a total of 12 specimens (sequences in the GenBank, accessions HM196012–HM196023). Also, the final list consisting of each identified potentially ectomycorrhiza-forming species was generated (Table 2). Species-level nomenclature follows Knudsen & Vesterholt (2008).

Ectomycorrhizal pine roots were collected in September 2004 using a soil corer (diameter 3 cm) in C, M, and OC areas. Across the experiment, a total of 300 soil cores were sampled. In each area, 10 focal trees adjacent to the plots were selected and 10 soil cores from the entire organic horizon (F and H layers of the podzol profile) were collected for a total of 100 soil cores in total. The core samples were transported to the laboratory in coolers within 8 h, and stored in $-20\,^{\circ}$C. The cores were thawed in groups of 10, and the soil was gently dry-sieved to separate the roots from soil. The cores were examined one by one until pine roots were found; a small number of cores contained no roots. The collected roots were carefully cleaned from adhering soil with sterilized water under a dissection microscope, and to avoid sampling the same 'fungal genotypes' several times (Lilleskov *et al*., 2004), one to three morphologically distinct root tips with a visible mantle were excised from each core sample, rinsed again in sterilized water, and stored at $-20\,^{\circ}$C in 1.5-mL tubes in water for DNA extraction. This sampling strategy resulted in a total of 191, 354, and 206 root tips from C, M, and OC areas, respectively.

The fungal in-growth bags (Wallander *et al*., 2001) were used to provide a more comprehensive view of the ectomycorrhizal diversity. Note that the in-growth bags primarily target the actively growing nonsaprophytic species during the incubation period (Wallander *et al*., 2001). The bags ($\sim 4 \times 12$ cm in size) were constructed of nylon mesh (mesh size 50 μm) and filled with 300 g acid-washed quartz sand. Two bags were placed horizontally under the humus layer in each of the 10 study plots at C, OC, and NC areas, and incubated for two growing seasons (2006–2007). Hyphae were then isolated by floatation from the sand and preserved in 1.5-mL microcentrifuge tubes at $-20\,^{\circ}$C for DNA extraction and further analysis.

DNA extraction

To provide genomic DNA from the collected sporocarps, total DNA was extracted from a few milligrams of dried gill tissue of each specimen using the NucleoSpin Plant kit (Macherey-Nagel). DNA from the root tips was extracted using the FastDNA SPIN Kit for soil (Qbiogene Inc.). Because our root tip samples were much smaller than the soil sample targeted in the manufacturer's protocol, the

Table 2. Ectomycorrhizal species found as sporocarps in 2003–2005 in a Pb-contaminated pine forest in Hälvälä

Order	Family	Species	C	M	OC
Agaricales	*Amanitaceae*	*Amanita muscaria*	+	+	+
Agaricales	*Amanitaceae*	*Amanita porphyria**	+		+
Agaricales	*Amanitaceae*	*Amanita spissa**		+	
Agaricales	*Cortinariaceae*	*Cortinarius aff. bataillei**	+		
Agaricales	*Cortinariaceae*	*Cortinarius aff. croceus**	+		+
Agaricales	*Cortinariaceae*	*Cortinarius biformis coll.**	+	+	+
Agaricales	*Cortinariaceae*	*Cortinarius gentilis*	+	+	
Agaricales	*Cortinariaceae*	*Cortinarius glandicolor*		+	
Agaricales	*Cortinariaceae*	*Cortinarius odhinnii*	+		
Agaricales	*Cortinariaceae*	*Cortinarius phoeniceus*	+		
Agaricales	*Cortinariaceae*	*Cortinarius semisanguineus*	+	+	+
Agaricales	*Cortinariaceae*	*Cortinarius traganus*	+		+
Agaricales	*Crepidotaceae*	*Inocybe soluta coll.**		+	
Agaricales	*Tricholomataceae*	*Laccaria laccata coll.**	+		
Agaricales	*Tricholomataceae*	*Tricholoma imbricatum**	+		
Agaricales	*Tricholomataceae*	*Tricholoma saponaceum**	+		
Boletales	*Boletaceae*	*Boletus pinophilus*	+		
Boletales	*Boletaceae*	*Chalciporus piperatus*	+		
Boletales	*Boletaceae*	*Leccinum scabrum coll.*	+	+	+
Boletales	*Boletaceae*	*Leccinum vulpinum*		+	
Boletales	*Gomphidiaceae*	*Chroogomphus rutilus*	+		
Boletales	*Paxillaceae*	*Paxillus involutus*	+	+	+
Boletales	*Suillaceae*	*Suillus bovinus*	+	+	+
Boletales	*Suillaceae*	*Suillus luteus*	+	+	+
Boletales	*Suillaceae*	*Suillus variegatus*	+	+	+
Boletales	*Suillaceae*	*Xerocomus badius* (*Boletus badius*)*			+
Cantharellales	*Cantharellaceae*	*Cantharellus cibarius*	+	+	+
Cantharellales	*Hydnaceae*	*Hydnum repandum*	+	+	
Russulales	*Russulaceae*	*Lactarius musteus**	+		
Russulales	*Russulaceae*	*Lactarius rufus*	+	+	+
Russulales	*Russulaceae*	*Russula decolorans*	+	+	
Thelephorales	*Thelephoraceae*	*Thelephora terrestris coll.**	+		

*Sequenced species.

C, control area; M, medium contaminated area; OC, heavily contaminated old shooting range area; +, at least one sporocarp is found.

protocol was modified as follows: half of the ceramic and silica particles were removed from the Lysing Matrix E tubes and one ectomycorrhizal root tip was added to the tube. Instead of the full volume of 500 μL, 245 μL sodium phosphate buffer and 30.5 μL MT buffer were added. Lysing Matrix E tubes were processed in the FastPrep Instrument for 60 s at speed 6. After that, 62.5 μL PPS was added and 125 μL cloudy Binding Matrix Suspension was used to bind DNA. One hundred and fifty microliters of SEWS-M was added to wash the filter. The final DNA was eluted in 20 μL of deionized water. DNA from the hyphae in the sandbags was extracted using a FastDNA SPIN Kit for soil (Qbiogene Inc.) as in the manufacturer's protocol.

PCR amplification

PCR amplification of the sporocarp DNA followed Niskanen *et al.* (2009). The primers internal transcribed spacer (ITS) 1F and ITS4 (White *et al.*, 1990; Gardes & Bruns, 1993) were used to amplify the ITS regions of the rDNA. The root tip DNA was amplified with a fungus-specific primer pair, FUN28f (Hultman *et al.*, 2010) and ITS4 (White *et al.*, 1990). The forward primer was designed to hybridize to the 3′-end of the 18S rRNA gene. Like the amplicons for 454 sequencing (below), the resulting amplicons span across the two ITS regions and the 5.8S rRNA gene. The PCR reactions were performed on GeneAmp PCR System 2700 (Applied Biosystem, Foster City, CA) using a 30 μL reaction volume containing 1 × PCR buffer [10 mM Tris-HCl (pH 8.8 at 25 °C), 1.5 mM $MgCl_2$, 50 mM KCl, and 0.1% Triton X-100 (Finnzymes, Espoo, Finland)], 200 μM each dATP, dTTP, dGTP, and dCTP, 0.3 μM primer, 1 U DNA polymerase (DyNAzyme©, Finnzymes), 0.0025 U *Pfu*-polymerase (Fermentas, Vilnus, Lithuania), and 3 μL DNA template. The cycling parameters included an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 30 s, 72 °C for 1 min 30 s, followed by a final extension

of 10 min at 72 °C. A negative control with no DNA template was included in each PCR. The quantity of the PCR products was checked by running a 5 μL sample of each PCR reaction on a 1% agarose gel at 150 V for 40 min. The gel was stained with ethidium bromide and visualized under UV light. If the PCR product was faint or showed no clear band, it was discarded.

Sequencing

The sporocarp DNA was sequenced with the PCR primers (above) as described in Niskanen *et al.* (2009). DNA from the ectomycorrhizal root tips was sequenced using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit v.3.0 and analyzed on an ABI Prism 3700 DNA sequencer (Applied Biosystems) in the DNA Sequencing Laboratory, Viikki Biocenter, University of Helsinki. Sporocarp DNA sequences were assembled and edited using SEQUENCER 4.1 (Gene Codes, Ann Arbor) and root tip sequences were compiled using the STADEN PACKAGE (Staden, 1996). Poor-quality terminal reads and poor-quality sequences were cleaned with PREGAP4 (Staden, 1996). Sequences were proof-read and corrected based on chromatograms in GAP4 (Staden, 1996). Sequences with an identity $\geq$99% were considered as operation taxonomic units (OTUs). The OTUs were aligned to the European Molecular Biology Laboratory (EMBL) database with FASTA (Persson & Lipman, 1988). A representative sequence for each root tip OTU has been submitted to GeneBank under accession numbers HM196024–HM196050.

The DNA from the in-growth bags was quantified using an ND1000 spectrometer (NanoDrop Technologies, Wilmington, Delaware) and adjusted to a final 2.5 ng μL^{-1} concentration. Similar to Jumpponen & Jones (2009), the ITS amplicons were 454-sequenced (Margulies *et al.*, 2005) using primer constructs that incorporated the 454-sequecing primers (A or B), sample-specific DNA tags, and ITS1F (Gardes & Bruns, 1993) or ITS2 primers (White *et al.*, 1990). Each sample was amplified in three separate 25 μL PCR reactions that contained final concentrations or absolute amounts of reagents as follows: 200 nM of each of the forward and reverse primers, 5 ng of the template DNA, 200 μM of each dNTP, 2.5 mM $MgCl_2$, 1 U of GoTaq Hot Start DNA polymerase (Promega, Madison, WI), and 2.5 μL of PCR buffer. The PCR cycle parameters consisted of an initial denaturation at 94 °C for 3 min, then 25 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, and extension at 72 °C for 2 min, followed by a final extension step at 72 °C for 10 min. All PCR reactions were performed in 96-well PCR plates on a MasterCycler (Eppendorf, Hamburg, Germany). Possible contamination was assessed by a blank sample run through the extraction and a negative PCR control in which the template DNA was replaced with sterile H_2O. These remained free of PCR amplicons.

A total of 15 μL of each of the three amplicons for each sample was pooled, purified using an AmPure SPRI (Agencourt Bioscience, Beverly, MA) magnetic PCR clean-up, and quantified (ND1000 spectrometer). For each sample, 100 ng was combined for sequencing and this pool was adjusted to 10 ng μL^{-1}. The pooled products were sequenced in a 1/16th region of a reaction of a GS FLX sequencer (454 Life Sciences, Branford, CT) at the Interdisciplinary Center for Biotechnology Research at University of Florida.

The raw, fasta-formatted sequences were searched for the DNA tag preceding the ITS2 primer. Sequences shorter than 200 bp, without a primer sequence or a DNA tag, or with ambiguous bases were excluded. Where present, the DNA tag was replaced with a sample designation, sequences aligned with CAP3 (Huang & Madan, 1999), and assigned to OTUs at 69–99% sequence identity at 2% intervals using a minimum overlap of 100 bp. All singletons as well as example sequences for each OTU at 99% similarity are available at GenBank (HM196051–HM196166). The data were parsed by sample to calculate the OTU frequencies for each sample.

Data analysis

The sporocarp and root tip data could not be analyzed statistically because of the limited numbers of observations in those data sets. Species lists were thus compared visually in order to determine possible similarities.

To analyze the 454 data, the OTUs were assigned at 95% sequence identity as suggested by Jumpponen & Jones (2009) and Jumpponen *et al.* (2010). A representative of each nonsingleton OTU was assigned to a genus, family, and order based on BLAST matches (Zhang *et al.*, 2000) after filtering out accessions that were annotated as environmental sequences or as unculturable fungi. Those OTUs that represented an ectomycorrhizal taxon were identified as such and other nontargets (22% of all sequences) were excluded from further analyses. OTU frequencies for each ectomycorrhizal taxon were summed across each experimental unit (study plot) and genus. The database annotations may be inaccurate (Nilsson *et al.*, 2006; Arnold *et al.*, 2007), but these assignments provide a useful taxonomic framework. To account for the effect of poor BLAST matches, we removed data points whose assignment was based on a short overlap ($<$ 80% coverage) or a low sequence identity ($<$ 90% similarity) from these analyses.

The richness and diversity indices of 454 data were calculated from the OTU frequency output. The overall OTU richness (S) was calculated by summing the number of OTUs, including singletons, within each sample. Simpson's dominance ($D = \Sigma p_i^2$), Simpson's diversity ($1 - \Sigma p_i^2$ and $1/\Sigma p_i^2$), and Shannon's diversity ($H' = -\Sigma p_i(\ln(p_i))$) were calculated for each sample, where p_i is the frequency of the occurrence of each OTU. A final index of diversity,

Fisher's α log-series (Fisher *et al.*, 1943), was calculated by iterating the equation $S/N = [(1-x)/x][-\ln(1-x)]$, where S is the richness and N is the total number of sequences within the sample. Once x was solved, the diversity index (α) was calculated as $N(1-x)/x$. To explore organismal coverage among the Pb contamination levels, species accumulation (rarefaction) curves and extrapolative richness estimators were generated using ESTIMATES (version 8; Colwell, 2006). Finally, these indices were compared using a one-way ANOVA model with 'area' as the independent variable that identified the timing or the level of Pb contamination in JMP (7.0.1; SAS Institute, Cary, NC).

The 454 data were further analyzed for the community structure of ectomycorrhizal fungi to examine the fungal community responses to Pb contamination (PC-ORD v. 4.1, McCune & Mefford, 1999). The soil data are the same as those published by Rantalainen *et al.* (2006). Pairwise distances were estimated using the Sørensen (Bray–Curtis) index and analyzed using nonparametric multidimensional scaling (NMS; Mather, 1976) multivariate analysis to avoid issues stemming from potential non-normality. The optimal number of dimensions (k) was selected based on the Monte Carlo test of significance at each level of dimensionality comparing 40 runs with empirical data against 50 randomized runs with a step-down in dimensionality from 6 to 1 and a random seed starting value. The k = three-dimensional solutions yielded similar results and produced solutions with stress values smaller than those in randomized runs ($P = 0.0196$). Accordingly, the three-dimensional solution was selected and the data were reordinated with a $k = 3$ configuration. To determine community differences among the Pb contamination levels, the NMS scores were analyzed using ANOVA as described above (JMP 7.0.1; SAS Institute).

Results

Sporocarps and root tips

We recorded a total of 32 species of ectomycorrhizal fungi that formed macroscopic epigeous sporocarps (Table 2). Half of them (16 species) represented *Agaricales* and a third (10 species) represented *Boletales*. A total of 22 species were recorded in the control area (C), 15 in M, and 21 in OC areas. Ten species occurred in all three areas. A total of 11

Table 3. Ectomycorrhizal species identified from the pine root tips in a Pb-contaminated pine forest in Hälvälä

Order	Family	Species	Reference	Similarity	C	M	OC
Agaricales	*Amanitaceae*	*Amanita spissa*	AJ889924	100	–	–	3
Agaricales	*Cortinariaceae*	*Cortinarius armeniacus*	DQ117925	99	–	–	2
Agaricales	*Cortinariaceae*	*Cortinarius biformis*	UDB002252	100	–	9	–
Agaricales	*Cortinariaceae*	*Cortinarius glandicolor*	DEU266700	100	4	–	3
Agaricales	*Cortinariaceae*	*Cortinarius melitosarx*	HM196016	100	–	9	5
Agaricales	*Cortinariaceae*	*Cortinarius sect. Dermocybe* sp.	AJ236061	100	1	2	–
Agaricales	*Cortinariaceae*	*Cortinarius* sp.	AY097036	98	–	–	6
Agaricales	*Cortinariaceae*	*Inocybe* sp.	DQ517413	98	–	8	–
Agaricales	*Entolomataceae*	*Nolanea conferenda*	AF538624	99	–	–	1
Atheliales	*Atheliaceae*	*Amphinema* sp.	FM992887	99	3	–	8
Atheliales	*Atheliaceae*	*Piloderma croceum*	PCR438982	99	5	1	1
Atheliales	*Atheliaceae*	*Piloderma fallax* 1	DQ365667	99	9	15	8
Atheliales	*Atheliaceae*	*Piloderma fallax* 2	AJ534903	99	8	–	–
Atheliales	*Atheliaceae*	*Piloderma* sp.	EF493279	99	7	30	16
Atheliales	*Atheliaceae*	*Tylospora asterophora*	AF052557	98	12	–	–
Atheliales	*Atheliaceae*	*Tylospora fibrillosa*	AF052563	99	1	–	–
Atheliales	*Atheliaceae*	*Tylospora* sp.	AB456677	98	–	1	–
Boletales	*Suillaceae*	*Suillus luteus*	AJ272413	100	–	1	–
Boletales	*Suillaceae*	*Suillus variegatus*	AM086444	100	4	3	–
Dothideomycetes		*Cenococcum geophilum*	AY112935	99	–	2	1
Russulales	*Russulaceae*	*Lactarius rufus*	DQ097868	100	32	35	14
Russulales	*Russulaceae*	*Russula betularum*	RBE534937	99	4	–	–
Russulales	*Russulaceae*	*Russula decolorans*	AY194601	99	10	–	–
Thelephorales	*Thelephoraceae*	*Thelephora terrestris* 1	FN393122	93	–	–	1
Thelephorales	*Thelephoraceae*	*Thelephora terrestris* 2	U83486	99	–	–	2
Thelephorales	*Thelephoraceae*	*Tomentella stuposa*	AY010277	99	–	2	–
Thelephorales	*Thelephoraceae*	*Tomentella sublilacina*	AF272935	99	1	4	–

Numbers indicate in how many of the root tips each species occurred.
Reference to EMBL databases with 93–100% similarity.
C, control area; M, medium contaminated area; OC, heavily contaminated old shooting range area.

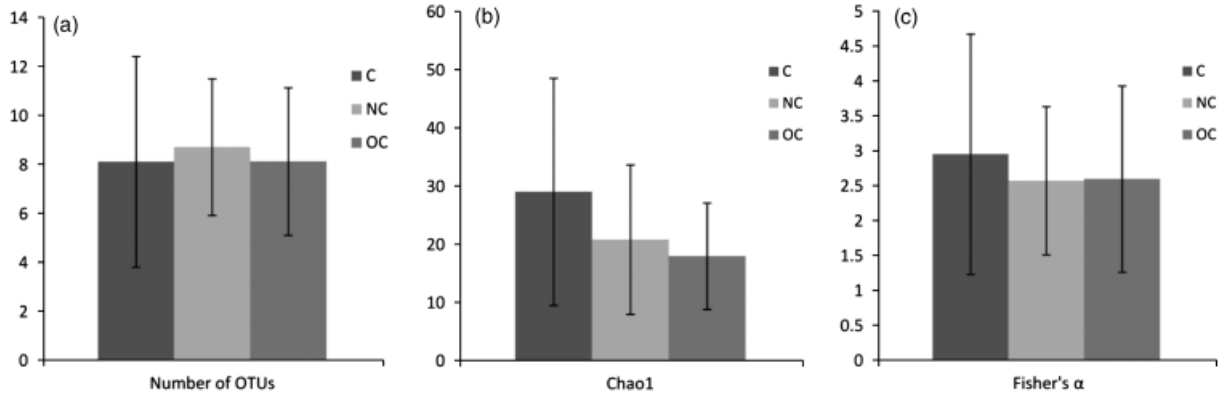

Fig. 2. (a) Number of OTUs, (b) Chao1 diversity index, and (c) Fisher's α diversity index for ectomycorrhizal fungi in control (C), new contaminated (NC) and old contaminated (OC) areas in the Hälvälä shooting range. Error bars indicate ± 1 SD, $n = 10$.

species that were found at C or M areas were absent in the OC area, and a total of three species occurred exclusively in the control area. Nine species were recorded only in either or both of the Pb-contaminated areas (Table 2).

After removing short (< 400 bp), poor-quality, or presumably chimeric sequences, the remaining 294 full-length ITS reads sequenced directly from the root tips included 27 OTUs that matched putative and well-annotated species in EMBL databases (Table 3) and were identified as ectomycorrhizal. One of them, *Russula betularum*, is considered to be associated with *Betula*, but because it was found from our root tip collection, it was included in the studies.

Eight species occurred in both sporocarp and root tip data (Tables 2 and 3). *Lactarius rufus* was the most abundant in the root tips, followed by *Piloderma* sp.; both of these taxa occurred in all three areas. *Suillus luteus* and *Suillus variegatus* fruited abundantly in all three areas, but were infrequent among the root tips. Two species of *Russula* and two species of *Tylospora* were found only in the C area root tips. An additional, unidentified *Tylospora* sp. was recorded exclusively in the M area. Several species occurred only in the OC area. For example, *Amanita spissa* and *Thelephora terrestris* showed this trend both in sporocarp and in root tip data. *Cortinarius* was the most species-rich genus both in sporocarp and in root tip data and did not show any clear trend on a genus or on a species level among the sites with differing degrees of Pb contamination.

Ectomycorrhizal fungi in the in-growth bags

To characterize the fungal communities in the in-growth bags, we 454-sequenced a total of 9527 amplicons. Our quality control excluded 1333 sequences as a result of lacking primer or DNA tag sequences or because they did not meet our sequence length threshold. The resulting final data set contained 8194 sequences, providing a sequencing depth of 152 ± 28 reads per sample (mean ± 1 SD). The number of sequences was invariable among the areas (ANOVA, $F_{2,30} = 0.94$; $P = 0.3957$), indicating that the samples were pooled accurately. The nonectomycorrhizal taxa were filtered after the BLAST-based assignments. After the removal of one low-yield sample in OC, the remaining numbers of ectomycorrhizal sequences remained invariable among the three areas (ANOVA, $F_{2,29} = 3.06$; $P = 0.0640$), indicating that conclusions derived from these analyses were not a result of bias from unequal sampling effort.

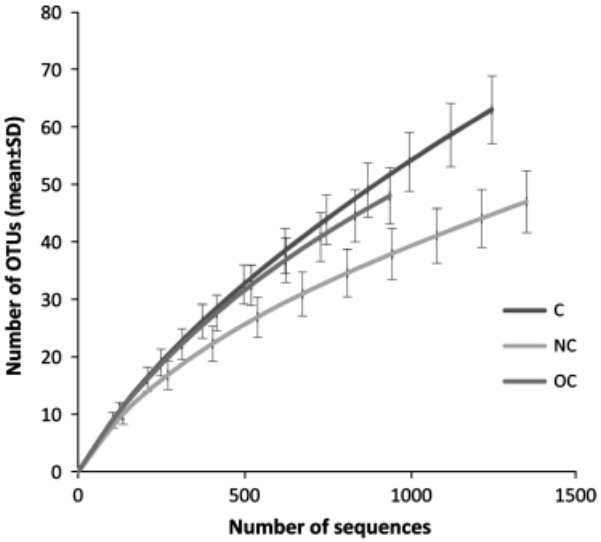

Fig. 3. Rarefaction curves for ectomycorrhizal fungi in control (C), new contaminated (NC), and old contaminated (OC) areas in the Hälvälä shooting range. OTUs were assigned at 95% similarity for these analyses. Error bars indicate ± 1 SD, $n = 10$.

In the in-growth data set, ectomycorrhizal sequences account for 50% of the number of OTUs (79/161 of nonsingleton), but 85.2% of the reads of our sequences corresponded to ectomycorrhizal fungi, indicating a diverse

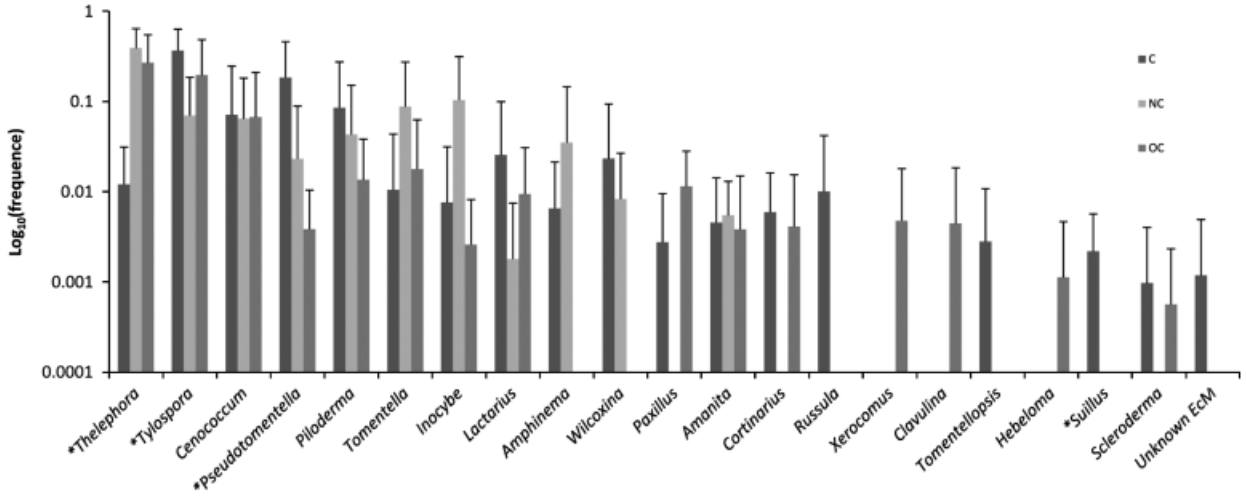

Fig. 4. Occurrence of 21 ectomycorrhizal genera in control (C), new contaminated (NC), and old contaminated (OC) areas at the Hälvälä shooting range. Error bars indicate $\pm$ 1 SD. *Significance of the frequency difference between areas at level $P \leq 0.05$; nonsignificant comparisons are not shown.

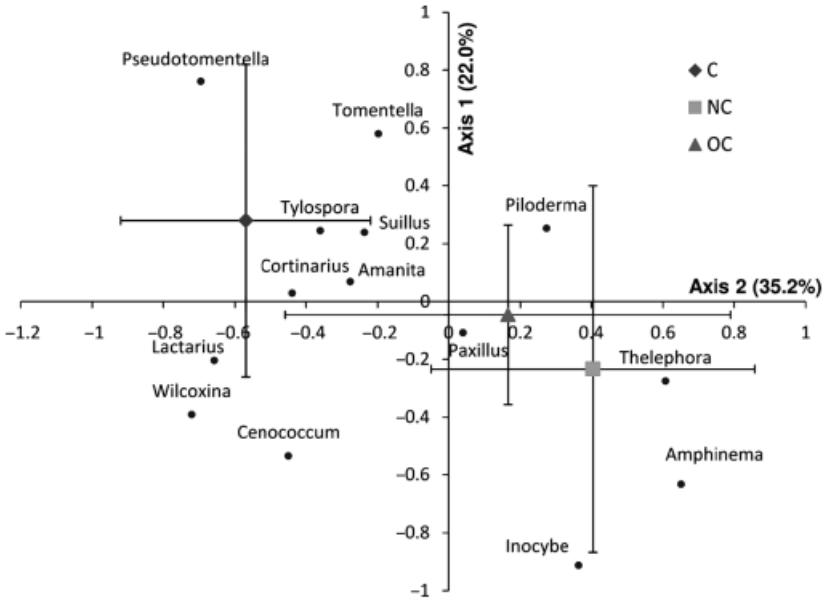

Fig. 5. NMS ordination of ectomycorrhizal fungus communities in the control (C), the new contaminated (NC), and the old contaminated (OC) areas at the Hälvälä shooting range. The scores (mean $\pm$ 1 SD) for the three areas are plotted. The *post hoc* test indicated that C is clearly different from NC and OC. The percentages following the axes indicate the proportion of variation represented by that axis. The *X*-axis is correlated with the Pb concentration of the soil. An ANOVA test of plot scores showed a significant difference among areas ($F_{2,29} = 10.7806$, $P = 0.0004$). Dot symbols represent the correlation of genera with axes.

community of nonectomycorrhizal fungi that occur at low frequencies. Ectomycorrhizal richness (number of OTUs, *S*) and diversity (Fisher's α, Simpson's $1/D$, and Shannon's H') were invariable among the three areas (Fig. 2; Supporting Information). Similarly, extrapolative richness estimators (Chao1, Jackknife; Fig. 2) indicated no differences among the areas with different Pb exposure histories. The rarefaction curves did not approach an asymptote (Fig. 3), suggesting that the total ectomycorrhizal richness in the ingrowth bags was inadequately covered due to the shallow sequencing and more OTUs remained to be detected with additional sequencing effort. Similarly, the extrapolative richness estimators exceeded the observed richness and suggested that, on average, only approximately 30–50% of the total, extrapolated richness was observed (compare Chao1 and *S* in Fig. 2a and b). However, it is worth noting that our goal was not to saturate the organismal richness in our samples, but to sample a representative number of experimental units to test hypotheses on the diversity and community differences of ectomycorrhizal fungi among the variously Pb-contaminated pine forest soils.

As expected, a majority of the fungi detected in the ingrowth bags were assigned to taxa that we considered ectomycorrhizal (6813 sequences; 85.2%). The remaining sequences represented putative ericoid (*Rhizoscyphus ericae*; two sequences; 0.03%) and arbuscular mycorrhizal taxa (*Glomus* sp.; four sequences; 0.05%) or were considered nonmycorrhizal (1177 sequences; 14.7%). Of the total 161

nonsingleton OTUs at 95% sequence identity, 79 were assigned to ectomycorrhizal taxa. On a genus level, these BLAST-assigned OTUs represented *Tylospora* (*Atheliaceae*; 1854 sequences; 27.2% of all ectomycorrhizal sequences), followed by *Thelephora* (*Thelephoraceae*; 1566 sequences; 23.0%), *Pseudotomentella* (*Thelephoraceae*; 595 sequences; 8.7%), *Cenococcum* (uncertain placement; 549 sequences; 8.1%), *Inocybe* (*Cortinariaceae*; 381 sequences; 5.5%), and *Piloderma* (*Atheliaceae*; 325 sequences; 4.8%). Ectomycorrhizal genera were ranked and their frequencies were compared by ANOVA in the three areas (Fig. 4). The results suggested that *Tylospora*, *Pseudotomentella*, and *Suillus* were significantly more frequent in the C area. In contrast, *Thelephora* occurred more frequently in contaminated areas.

To summarize and visualize the compositional differences in the ectomycorrhizal communities among the three areas, we used NMS multivariate analyses. The two first ordination axes represented 57.2% of the variation and separated the ectomycorrhizal communities in the control and the contaminated areas (Fig. 5). Genera were broadly distributed in the ordination space, indicating that the ectomycorrhizal fungal community existed in the environment along a wide Pb contamination range. *Wilcoxina*, *Pseudotomentella*, and *Lactarius* most clearly showed a negative correlation and *Thelephora* and *Amphinema* a positive correlation with Axis 2, indicating their contrasting occurrences in the three study areas and supporting the potential greater occurrence of the latter two taxa in the Pb-contaminated areas.

Discussion

We assayed ectomycorrhizal communities in a Pb-contaminated Scots pine forest site using three different approaches: sporocarps, ectomycorrhizal roots, and in-growth bags. None of these showed any strong change in diversity or richness in the ectomycorrhizal fungal community when Pb-contaminated areas were compared with the control. Still, we observed a shift in the ectomycorrhizal community composition, which was supported by all three approaches. The community shifts were largely attributable to responses of four taxa in the in-growth bags: an increase in *Thelephora* and a decrease in *Pseudotomentella*, *Suillus*, and *Tylospora*. Similar to our results, Moser *et al.* (2005) reported that, despite the differences in the community composition, the ectomycorrhizal fungal richness and diversity did not differ between the Pb-rich serpentine soils and control soils. The results from our present study also support our previous research: high Pb loads affect various components of a boreal forest ecosystem, including the fungal communities (Hui *et al.*, 2009), fungal biomass (Rantalainen *et al.*, 2006), and the fungal enzyme activities (Tuomela *et al.*, 2005).

The various diversity indices estimated for the in-growth bag ectomycorrhizal communities showed no difference between the sites. The Pb tolerances may vary strongly among ectomycorrhizal fungal species (Hartley *et al.*, 1997) and only the sensitive species may be strongly affected by Pb. Even if Pb may have reduced the number of sensitive ectomycorrhizal species in our Pb-contaminated study sites, these may have been replaced by insensitive ones, leaving the diversity and richness estimators unchanged. It is also possible that the unchanged diversity and richness may have been facilitated by the spatial variability in the Pb contamination, which ranged from 2.2 to 50 $g\,kg^{-1}$ (Rantalainen *et al.*, 2006). In this case, sensitive species may persist in minimally contaminated patches, thus responding via the spatial distribution and patchiness rather than the richness and diversity. Finally, ectomycorrhizal fungi rarely occur in the environment without their symbiotic host plants. Heavy metal tolerances of ectomycorrhizal fungi, including Pb-sensitive species, have been reported to be higher in symbiosis than when the fungi were grown alone without their hosts (Jones & Hutchinson, 1986; Colpaert & van Assche, 1993).

Examination of the correlation between the community scores and the Pb concentrations in our NMS analyses provided an additional insight into the ectomycorrhizal community structuring. The NMS results were not fully consistent with our genus-level comparisons utilizing ANOVA. While the comparisons of frequencies using ANOVA identified *Thelephora* as positively and *Pseudotomentella*, *Suillus*, and *Tylos*pora as negatively affected genera, our NMS analyses showed positive correlations between Pb contamination and – not only *Thelephora* – but also *Amphinema*. Similarly, *Pseudotomentella*, *Lactarius*, and *Wilcoxina* were negatively correlated with increasing Pb contamination, but only *Thelephora* and *Pseudotometella* showed a significant difference between areas in ANOVA.

Pb tolerance or sensitivity of the observed taxa is poorly known. The tolerance of *T. terrestris* and the sensitivity of *Pseudotomentella* were supported by both analytical approaches supporting the contrasting responses of these fungi to Pb. *Thelephora terrestris* strains have also shown tolerance to high Pb concentrations in liquid cultures, although this may be partly attributable to the precipitation of Pb in the higher concentration ranges of Pb in the liquid media (Tam, 1995). In contrast, McCreight & Schroeder (1982) reported that the growth of *T. terrestris* on agar media was arrested when the Pb concentration was 200 $\mu g\,mL^{-1}$. These results suggest that *T. terrestris* is able to tolerate Pb only to some degree in a pure culture. In our study, both root tip and in-growth bag data revealed that *Thelephora* was most frequent in a Pb-contaminated area. This discrepancy between our field observations and the laboratory tests may stem from the high genotypic variation in the field: while our experiment observed multiple genotypes assigned to OTUs by BLAST, many laboratory studies have used only limited numbers of strains. The sensitivity of *Pseudotomentella* seems to be clear in our data, but there are no references in

support. *Tylospora*, belonging to *Thelephoraceae*, showed opposite behavior, decreasing in frequency in contaminated areas compared with the controls (ANOVA). *Wilcoxina* tends to be a generalist fungus with an extensive geographical distribution and a wide ecological range (Mikola, 1988). It has also been found in heavy metal-contaminated soils, but our data rather indicate Pb sensitivity.

Most abundant species in our study represented taxa known to be common in boreal pine forests: *Lactarius*, *Suillus*, and *Russula* (Väre *et al.*, 1996). *Lactarius* was the most abundant taxon in the pine roots, common among sporocarps and frequent in the in-growth bags. *Suillus* generally tends to form large numbers of sporocarps and tends to be abundant in root tips in coniferous forests (Gardes & Bruns, 1996; Dahlberg, 2002). However, this was not true for our study site. Although frequent among the sporocarps, it was infrequent among the root tips or in the in-growth bags. *Russula* occurred most frequently in the control area in our study, but was also found at lower frequencies in all areas. Because there are no published data about its response to Pb contamination, we can suggest this genus as Pb sensitive only with great caution.

The three different assay methods produced incongruent results. There are several possible reasons for this. A large majority of ectomycorrhizal fungi do not fruit regularly, if at all, or they produce inconspicuous sporocarps that are hard to detect (Gardes & Bruns, 1996; Jonsson *et al.*, 1999; Dahlberg, 2001; Horton & Bruns, 2001; Tedersoo *et al.*, 2003). For example, genus *Piloderma* is typically very common in pine forests in Finland (Väre *et al.*, 1996), but forms inconspicuous, resupinate sporocarps. We detected this taxon frequently in the root tips and in the in-growth bags.

Additional incongruence between the data sets may arise from the seasonality of the fungal communities. Typically, few ectomycorrhizal taxa account for the greatest proportion of the mycorrhizal biomass and the contribution of most taxa is small (Horton & Bruns, 2001). Our fruiting body data contained only eight common species. The incongruence observed was especially striking for the species of *Cortinarius*, the most species-rich genus in our sporocarp and root tip data. Species occurring as sporocarps and root tips showed nearly no overlap.

The number of ectomycorrhizal species in boreal coniferous stands tends to exceed 50 within a study (Horton & Bruns, 2001). Our data with 57 recorded taxa in sporocarp and root tip data do not seem unrepresentative. Still, the differences in species composition in each of our data sets indicate that our sampling was inadequate to capture the entire community. Although we detected 116 ectomycorrhizal OTUs in the in-growth bag data set, the number of OTUs in each sample ranged from 2 to 17. This leads to the average extrapolative estimates that are more than twofold higher. Further, these estimators tend to be negatively biased if the total richness substantially exceeds the observed richness or when the sampling inadequately represents the communities (Palmer, 1990; Baltanas, 1992). The rarefaction analyses suggested insufficient sampling to saturated organismal coverage in our study area.

Conclusion

Our results show that although organismal diversity and richness seemed unresponsive to long-term Pb contamination, the ectomycorrhizal fungal communities shifted in composition. This was a trend visible in the sporocarp and root tip data and appeared to be statistically significant in the in-growth bag data. These compositional shifts were likely a result of an increase in the abundance of *Thelephora* and decreases in the abundances of *Pseudotomentella*, *Suillus*, and *Tylospora* in contaminated areas relative to the control. However, it remains unclear whether these community shifts have functional consequences for the dominant trees or soil ecosystem functions. Although our studies were conducted at one shooting range in Southern Finland, we expect these observations to be representative of similar general patterns in Pb-contaminated pine forest soils. However, further researches both in the field and in the laboratory are necessary to substantiate these results as well as to confirm the increase in the frequency of genus *Thelephora* in Pb-contaminated soils.

Acknowledgements

This study was supported by the Helsinki University Centre for Environment (HENVI), the Finnish Cultural Foundation, and the Finnish Graduate School in Environmental Science and Technology (EnSTe). Additional funding was received from the Elite project funded by ERDF and the Regional Council of Päijät-Häme.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. (a) Simpson's 1/*D* diversity index (b) Jack knife diversity index, and (c) Shannon's H' diversity index in control, new contaminated, and old contaminated areas. Error bars indicate $\pm$ 1 SD.

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