

# The influence of the land use on abundance and diversity of soil fungi: comparison of conventional and molecular methods of analysis

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## Abstract

This study examined fungal communities in 11 soil profiles. The objective was to apply conventional and molecular microbiology methods to obtain baseline information on the general characteristics of soil microbial communities in relation to land use – forest, former agricultural land, meadow and arable land. The number of filamentous fungi species and the total number of cultivable microorganisms showed a tendency to decrease with increasing depth. The diversity of fungi obtained with amplified rDNA gene restriction analysis was similar for all studied land use groups, diversity decreased with soil depth, especially in soils of former agricultural land and meadow. The proportion of fungal DNA as part of the total soil DNA was significantly higher in forest and abandoned land soil than in agricultural soil. The amount of *Trichoderma* spp. DNA was similar for all land groups, but its relative amount as percentage of total fungal DNA was higher in meadow and arable land soil. The land use type had a significant impact only on the diversity of cultivable soil fungi and fungal DNA amount. Soil depth and moisture content had a greater effects.

**Key words:** ARDRA, fungal diversity, Shannon-Weaver diversity index, soil, quantitative PCR, *Trichoderma*.

**Abbreviations:** AME, agarised malt extract; ARDRA, amplified ribosomal DNA gene restriction analysis; CFF, cultivable filamentous fungi; qPCR, quantitative PCR; CFU, colony forming units.

## Introduction

Soil is a heterogeneous environment and different components of the solid fractions in soil (sand, silt, clay, organic matter etc.) provide variable microhabitats. Major interrelated factors affecting microbial diversity in soil include soil forming processes, physicochemical properties of soil, soil particle size distribution, vegetation and land use type (management). The relative effects of these factors differ in different soil types, horizons and climatic zones (Garbeva et al. 2004). Several studies investigating the impact of different plant cultures and soil types have shown, that the soil type has the greatest influence on the structure of microbial populations (Chiarini et al. 1998; Grayston et al. 1998; Buyer et al. 1999; Marschner et al. 2001; Wieland et al. 2001), although under some conditions the influence of the vegetation may prevail, e.g. the maize rhizosphere accommodates specific bacterial communities, the structures of which change with the developmental state of the plant (Baudoin et al. 2002). Many publications

emphasize the influence of the land use (agricultural land, grassland, tropical forest and pasture, etc.) on the structure of soil microbial communities (Nusslein, Tiedje 1999; McCaig et al. 2001; Webster et al. 2002; Clegg et al. 2003; Drijber et al. 2003).

Abiotic conditions of soil (pH, moisture content and temperature) affect soil microorganisms in all soil land use types (Jurgensen et al. 1997; Hackl et al. 2004; Setälä, Mc Lean 2004; Borken et al. 2006; Ruisi et al. 2007; Zachow et al. 2009). It is known that fungi in soil constitute a significant part of the soil biomass and they have several important functions in soil, such as decomposition of organic material, nutrient cycling, formation of soil aggregates and mycorrhizal symbiosis (Kabir et al. 2003). Most (more than 80 %) soil fungi and bacterial species are not cultivable under laboratory conditions (Leckie 2005).

Two main approaches can be employed to study soil microbial communities – conventional plating of cultivable microorganisms and molecular methods that are independent of cultivation. Both approaches are associated

with specific advantages and disadvantages. Only a small portion of all microorganisms in soil can be cultivated in standard laboratory conditions, and the isolation of pure cultures and lengthy effort are needed for the determination of taxonomic identity of the isolates. In contrast, molecular methods provide general insight into the genetic heterogeneity of soil microbial communities and allow to identify specific microorganisms without isolation, but they are highly dependent on the success of the isolation of DNA from soil, eventual presence of the DNA amplification or restriction inhibitors, choice of the primers, discriminating power of analysis etc. (Kowalchuk et al. 2006).

Amplified ribosomal DNA gene restriction analysis (ARDRA) can be used for genetic fingerprinting of simple communities, populations or phylogenetic groups. High resolution discrimination can be obtained at the species level. In soil microbiology this method is used to determine diversity within phylogenetic or functional groups of microorganisms (Lynch et al. 2004). Digested PCR products can be run in polyacrylamide (Perez-de-Mora 2006) or in agarose gel (Smit et al. 1997; Schwieger, Tebbe 2000; Chabrerie et al. 2003). Usually at least two restriction enzymes are used and the obtained fragments can be analyzed as separate (Schwieger, Tebbe 2000; Klammer, Hedlund 2004) or combined (Wang et al. 2008) data sets.

The abundance of the specific groups of microorganisms in soil can be determined by quantitative PCR (qPCR) (Filion et al. 2003; Kabir et al. 2003; Kolb et al. 2003; Smits et al. 2004; Fierer et al. 2005). This method has been employed to quantify amounts of the genus *Trichoderma* fungi in soil by estimating their potential to counteract the activity of fungal plant pathogens (Cordier et al. 2006).

The objective of our investigation was to apply conventional and molecular microbiology methods in order to obtain baseline information about general characteristics of Northern temperate zone soil microbial communities depending on land use – forest, abandoned (former) agricultural land, meadow and arable land. We characterized and compared soil microbial communities employing conventional plating and molecular methods, ARDRA and qPCR. The number of cultivable microorganisms (colony forming units, CFU) was determined using the plate count method. Abundance of representatives of the typical fungal genera was determined by light microscopy of colonies. A robust culture-independent ARDRA and qPCR with universal fungal primers and *Trichoderma* spp. specific primers were used as molecular methods. The Shannon-Weaver diversity index ( $H'$ ) was determined for the communities.

## Materials and methods

### Sampling plots, soil sampling and estimation of soil pH

Samples from soil profiles or outcrops were taken with an auger from the Ap, A1 and B horizons of agricultural soils

and O, Ah and B horizons of forest soils (0 – 10 cm, 10 – 30 cm and 30 – 40 cm deep), on August 2007 in Jelgava district (Latvia), and on October 2007 in the Cesis and Valka districts (Latvia). The sampling sites were divided in the four groups: forest land; former agricultural land; agricultural land – meadow; agricultural land – conventional arable land. The characteristics of the soil profiles are given in Table 1. Samples were placed in sterile plastic bags (Nasco WHIRL-PAK) and stored at 4 °C for a few days until plating of cultivable microorganisms and later stored at –20 °C. The pH of the soil samples was measured in distilled water (ISO 10390). The moisture content of the soil was determined according to ISO 11465.

### Extraction and quality control of total soil DNA

Total soil DNA was extracted from the collected samples by harsh lysis using the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc.). Samples (250 mg) were homogenized using a horizontal Mixer Mill Type MM 301 (Retsch) at 1800 oscillations min<sup>-1</sup> for 10 min. DNA from each sample was extracted twice. The amount and purity of the DNA was assessed by spectrophotometry (Ultrospec 3100 Pro; Amersham Biosciences) and by 1% agarose gel electrophoresis.

### PCR and ARDRA of soil DNA

The fungal ribosomal RNA gene region containing two internal transcribed spacers and the 5.8S rRNA gene (ITS1-5.8S-ITS2) was amplified with primers ITS1F and ITS4 (Gardes, Bruns 1993). These primers amplify the ITS1-5.8S-ITS2 region of *Ascomycota*, *Basidiomycota* and *Zygomycota* fungi. Soil samples were analyzed undiluted, or in presence of some inhibitors in 1:10 and 1:100 dilutions.

In a Eppendorf Personal Mastercycler the reactions were carried out in 50 µL with 2 u of Hot Start *Taq* DNA Polymerase in 1× Hot Start PCR Buffer containing 0.2 mM each NTP, 8 mM MgCl<sub>2</sub> (Fermentas), 0.5 µM each primer (OPERON Biotechnologies) and 1 µL of DNA template. The PCR conditions were: 4 min at 95 °C, (40 s, 95°C; 40 s, 52 °C, 60 s, 72 °C) × 30 cycles and 10 min at 72 °C.

The PCR amplification products were precipitated by 450 µL of 96% ethanol and 3 M sodium acetate, pH 5.0 (19:1). After 15 min incubation at –20 °C, samples were centrifuged in a Sigma 1-15P centrifuge for 15 min at 14 000 rpm in room temperature, washed with 70% ethanol, dried and dissolved in 16 µL of sterile distilled water. The DNA was divided in two parts and digested with restriction endonucleases *Bsu*RI (Chabrerie et al. 2003) and *Eco*RI (Fermentas) separately. Restriction products were run in 2% agarose gels, photographed with a BioSpectrum AC Imaging System and analyzed with software KODAK1D. For the estimation of the Shannon-Weaver diversity index ( $H'_{ARDRA}$ ) the following equation was used:

$$H'_{ARDRA} = -\sum p_j \log_2 p_j$$
, where  $p_j$  is a relative intensity of individual band (Gabor et al. 2003).

**Table 1.** Soil profiles and their characterization. <sup>1</sup>The average pH ( $\pm$  SD) calculated from the pH measurements of all analyzed depths. <sup>2</sup>Sampling date 26.10.2007, average air temperature 4.02 °C. <sup>3</sup>Forest type according to the classification of Buss (1997). <sup>4</sup>Sampling date 27.10.2007, average air temperature 5.23 °C. <sup>5</sup>Sampling date 07.08.2007, average air temperature 20.61 °C. <sup>6</sup>Sampling date 08.08.2007, average air temperature 22.74 °C

Location	Soil profile	Soil type	Vegetation	Number of soil samples	pH	Soil moisture content at each depth (%)
Forest lands						
Cesis district, Taurene <sup>2</sup>	Forest1	Typical podzol	Haplic Cambisols Myrtillosa forest type – spruce ( <i>Picea abies</i> ), pine ( <i>Pinus sylvestris</i> ), birch ( <i>Betula pendula</i> ), gray alder ( <i>Alnus incana</i> )	3	5.12 $\pm$ 0.23	42.5; 8.4; 8.0
Valka district, Strenči <sup>4</sup>	Forest2	Typical podzol	Haplic Cambisols <i>Vacciniosa</i> forest type – spruce ( <i>Picea abies</i> ), pine ( <i>Pinus sylvestris</i> ), birch ( <i>Betula pendula</i> )	3	5.30 $\pm$ 0.53	6.6; 3.0; 8.2
Valka district, Strenči <sup>4</sup>	Forest3	Typical podzol	Haplic Arenosols <i>Cladinoso-callunosa</i> forest type – pine ( <i>Pinus sylvestris</i> ) monoculture	3	4.46 $\pm$ 0.62	4.5; 2.9; 45.0
Former agricultural lands						
Cesis district, Taurene <sup>2</sup>	Former1	Eroded soil	Haplic Luvisols Natural afforestation with spruce ( <i>Picea abies</i> )	3	7.53 $\pm$ 0.20	15.7; 19.3; 21.4
Cesis district, Taurene <sup>2</sup>	Former2	Colluvial soil	Haplic Luvisols Natural afforestation with spruce ( <i>Picea abies</i> )	3	6.68 $\pm$ 0.29	18.1; 17.6; 22.6
Agricultural lands, meadows						
Jelgava district, Svete <sup>5</sup>	Meadow1	Gleyic sod-podzolic soil	Stagnic Cambisols Regularly cut and pastured	3	7.90 $\pm$ 0.31	10.2; 10.6; 8.3
Jelgava district, Svete <sup>5</sup>	Meadow2	Granular alluvial soil	Fluvic Cambisols Regularly cut and pastured	3	7.44 $\pm$ 0.26	14.1; 13.2; 12.9
Jelgava district, Svete <sup>5</sup>	Meadow3	Pseudogley sod-podzolic soil	Endogleyic Umbrisols Regularly cut and pastured	2	7.72 $\pm$ 0.06	10.7; 10.4
Agricultural lands, conventional arable lands						
Jelgava district, Svete <sup>6</sup>	Field1	Pseudogley sod-podzolic soil	Endogleyic Umbrisols Arable land	2	7.55 $\pm$ 0.07	10.4; 9.8
Jelgava district, Svete <sup>6</sup>	Field2	Sod-pseudogley soil	Mollic Luvic Stagnosols Arable land	3	7.69 $\pm$ 0.11	14.2; 12.7; 10.9
Jelgava district, Svete <sup>6</sup>	Field3	Sod-gleyic soil	Endogleyic Umbrisols Arable land	2	6.96 $\pm$ 0.06	10.6; 13.2

### Analysis of cultivable microorganisms

In addition to the utilized molecular methods, cultivable microorganisms in three replicates were obtained from soil samples from forest and former agricultural land to estimate the CFU of filamentous fungi, bacteria and yeasts. Soil sample serial dilutions were prepared (Pepper et al. 1995; Alef, Nannipieri 1998). Agarised malt extract (AME) (30 g L<sup>-1</sup>, pH 5.4 ± 0.2, Biolife) was used as a growth medium, as it supports growth of filamentous fungi, yeasts and some groups of bacteria. In our work, the total number of cultivable microorganisms was estimated as the number of CFU of the microorganisms per gram of dry soil on AME after 120 h at 20 ± 2 °C (Vanderzant, Splittstoesser 1992).

Genera of cultivable filamentous fungi (CFF) were identified using keys (Barnett 1957; Kiffer, Morelet 2000) and light microscopy of the morphology of pure cultures.

For the estimation of the Shannon-Weaver diversity index of CFF ( $H'_{CFF}$ ) the following equation was used:

$H'_{CFF} = -\sum p_j \log_2 p_j$ , where  $p_j$  is a relative abundance of particular genera of CFF.

### Quantitative PCR

In addition to the methods described in previous chapters, total soil DNA from the upper horizons was subjected to qPCR. In qPCR either the ITS1-5.8S-ITS2 rDNA region of higher fungi with primers ITS1F and ITS4 or of *Trichoderma* spp. with the primers uTr and uTf (Hagn et al. 2007) was used to determine the amount of total fungal DNA and *Trichoderma* spp. DNA as part of the total soil DNA. Each soil sample was analyzed in three replicates.

In a SmartCycler (Cepheid) the reactions were carried out in 25 µL containing 12.5 µL SYBR® Premix Ex Taq (TaKaRa), 1 µM of each primer and 1 µL of the DNA template. The PCR conditions were: 30 s at 95 °C, (30 s, 95 °C; 30 s, 55 °C for primers ITS1F and ITS4 or 60 °C for primers uTr and uTf; 60 s, 72 °C) × 35 cycles for primers ITS1F and ITS4 or × 40 cycles for primers uTr and uTf. To quantify fungal DNA, qPCR using serial dilutions of DNA from pure cultures of *Heterobasidion parviporum*, *Penicillium lanoso-viride* MSCL 1 and *Trichoderma harzianum* MSCL 309 were performed and standard graphs were built (Fig. 1A). The average values of all three cultures were used for the calculation of the calibration curve for the total amount of the fungal DNA in soil. A standard graph (Fig. 1B) using serial dilutions of DNA of *T. harzianum* was used for the calculation of the concentration of trichodermal DNA in soil. In order to estimate the total copy number of the fungal DNA we assumed that the average fungal genome size was 35 Mb (Gregory et al. 2007) and that the average trichodermal genome size was 36.5 Mb (Kullman et al. 2005).

### Statistical analysis

In order to characterize the influence of human activity upon soil microbial communities we classified the soil types in four land use groups according to the increasing intensity of human impact: 1, forest land; 2, former agricultural land; 3, meadow; 4, agricultural land. The F-test, t-test ( $\alpha = 0.05$ ) and correlation analysis were conducted using MS Excel, and the program R was used for multiple regression analysis.

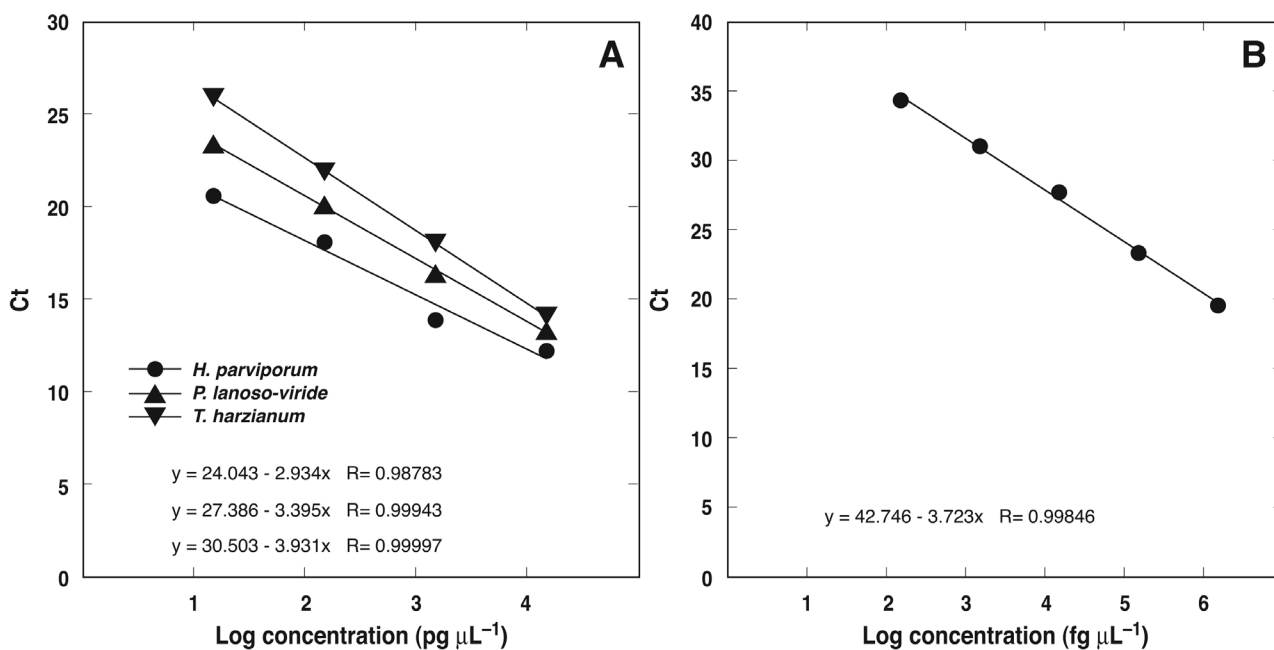
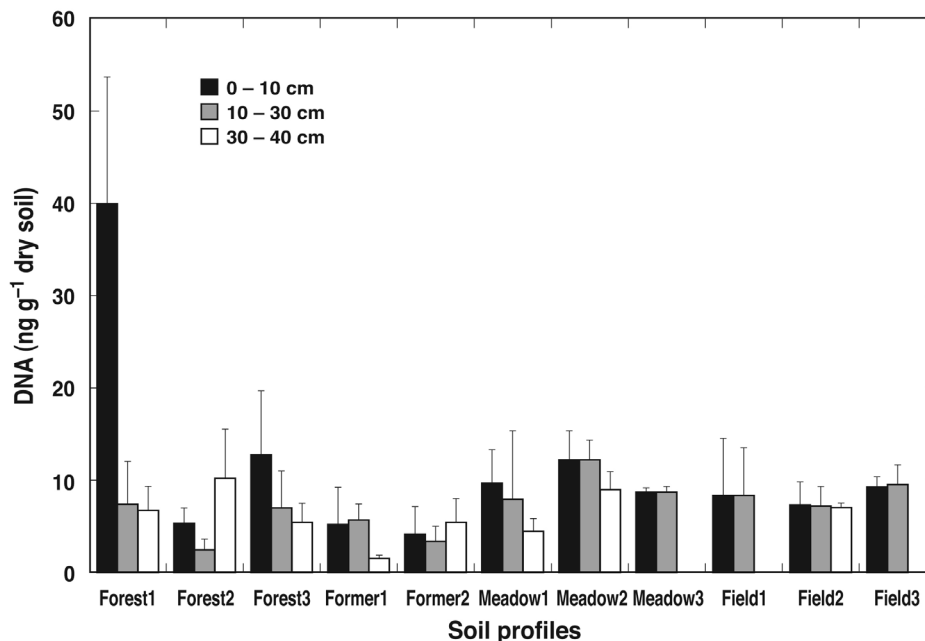


Fig. 1. Standard graphs for qPCR with *H. parviporum*, *P. lanoso-viride* and *T. harzianum* DNA dilution series with universal fungal primers (A) and standard graph with *Trichoderma harzianum* DNA dilution series with primers uTr and uTf (B).



**Fig. 2.** Total soil DNA amount (mean  $\pm$  SD,  $n = 2$ ). Total soil DNA was extracted from 0.25 g of soil using PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc.).

## Results

### The amount of total soil DNA

The amount of total soil DNA ranged from 1.53 in deeper soil layers of former agricultural lands up to 30.28  $\mu\text{g g}^{-1}$  of dry soil in upper horizons of forest soils (Fig. 2). The  $A_{260}/A_{280}$  ratio ranged from 1.6 to 1.8. In agarose gel electrophoresis the isolated DNA migrated as a diffuse band of a high molecular weight ( $>11$  kb) region (data not shown).

### Results of ARDRA

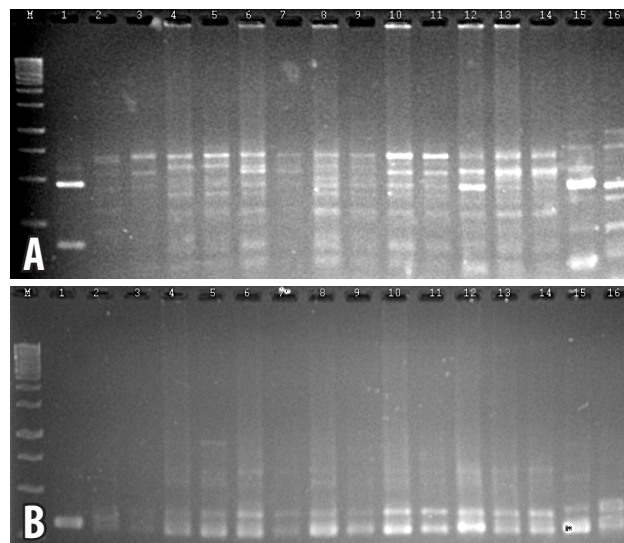
Typical ARDRA gels are shown in Fig. 3. We combined  $H^p_{ARDRA}$  results from both restriction reactions (Fig. 4) as suggested by Wang et al. (2008).

Only some soil profiles (Forest1, Former2, Meadow1, Meadow3 and Field2) showed significant differences (t-test) in  $H^p_{ARDRA}$  values between samples from different soil depths. In all soil profiles, the highest diversity of fungi was observed in the upper soil layer, with the exception of profiles Forest2 and Field2. In those two profiles the highest  $H^p_{ARDRA}$  was observed at a depth of 10 – 30 cm. In profile Forest3 the fungal diversity was significantly higher ( $p = 0.003$ ) than in the other two forest profiles.

In all the land use types, the mean fungal diversity  $H^p_{ARDRA}$  was similar for any of the examined sampling depths. In the 0 – 10 cm depth the  $H^p_{ARDRA}$  varied from  $2.61 \pm 0.56$  till  $3.04 \pm 0.24$ . At depth 10 – 30 cm, a significantly ( $p = 0.002$ ) lower diversity occurred in meadows compared to other land use types –  $2.26 \pm 0.08$  vs.  $2.73 \pm 0.29$ . In the deepest layer the  $H^p_{ARDRA}$  varied from  $1.95 \pm 0.57$  to  $2.48 \pm 0.48$ .

### The number of cultivable microorganisms, dominant fungal genera and diversity of CFF

The number of CFF in soil samples (Fig. 5) from forest and former agricultural soils decreased significantly ( $p < 0.05$ ) with increasing depth of sampling seen by comparing the upper soil layer (0 – 10 cm) with deeper layers (10 – 30 cm and 30 – 40 cm). The total number of CFU of



**Fig. 3.** Examples of ARDRA results in 2 % agarose gels. A – restriction with *Bsu*RI. B – restriction with *Eco*RI. Lanes in gels A and B: M-Gene Ruler 1 kb DNA Ladder (Fermentas). 1. Positive control with *T. harzianum* DNA. 2. Forest1, 30-40 cm. 3. Forest2, 0-10 cm. 4. Meadow2, 0-10 cm. 5. Meadow2, 10-30 cm. 6. Meadow3, 0-10 cm. 7. Meadow3, 10-30 cm. 8. Field1, 0-10 cm. 9. Field1, 10-30 cm. 10. Field2, 0-10 cm. 11. Field2, 10-30 cm. 12., 13. Field2, 30-40 cm. 14. Field2, 0-10 cm. 15., 16. Field2, 30-40 cm.

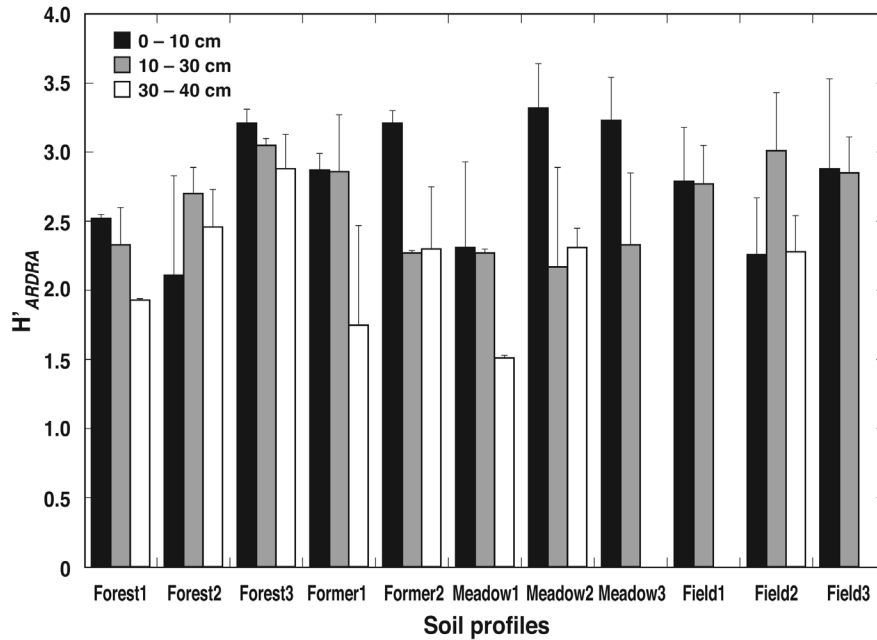


Fig. 4. Average Shannon-Weaver diversity index  $H'_{ARDRA}$  of fungal diversity in all analyzed land use groups (mean  $\pm$  SD, n = 2).

microorganisms also decreased with increasing depth of sampling in soil profiles Forest1, Forest3 and Former1 (Fig. 6), but not in profiles Forest2 and Former2. In profile Forest1 the differences were statistically significant ( $p < 0.05$ ) for all of the soil levels. In contrast to the other soils, profile Forest2 had a significantly ( $p < 0.05$ ) increased number of cultivable microorganisms at a depth of 30 – 40 cm in comparison to the depth 0 – 30 cm. In soil profile Former2 the total number of cultivable microorganisms was similar in all horizons.

In general the amount of CFF was higher (statistically not significantly) in forest land soil in comparison with former agricultural lands, but the total count of CFU of microorganisms was higher (not significantly) in former agricultural land soil (Fig. 5, Fig. 6).

The most abundant genera and groups of fungi in soils are listed in Table 2. The identified fungi belonged either to *Ascomycota* or *Zygomycota*. Sterile mycelia and also *Penicillium* and *Mucor* were in all samples. Sterile mycelia were abundant in all of the analyzed profiles. In deeper soil layers of Forest1, Forest2 and Former2 profiles, sterile mycelia made up more than 90% of CFF.

$H'_{CFF}$  index tended to be higher (not significantly) in forest soils than in former agricultural soils (Fig. 7).

#### Results of quantitative PCR

The highest amount of fungal DNA was found in forest soils, and the lowest in arable lands (no significant difference). The proportion of fungal DNA of the total soil DNA was the highest in the forest lands and former agricultural lands and lowest in meadows and fields (differences between these two groups was significant,  $p = 0.02$ ). The amount of *Trichoderma* spp. DNA was similar in all the land use types,

but the proportion of *Trichoderma* spp. DNA was higher in meadows and arable lands, in comparison to the other land use types,  $p = 0.0006$  (Table 3).

#### Results of statistical analysis

According to multiple regression analysis (Table 4), sampling depth significantly affected all analyzed parameters; the number of CFF, total number of microorganisms,  $H'_{CFF}$ ,  $H'_{ARDRA}$  and total soil DNA amount significantly decreased with depth. The strongest correlation was observed for number of CFF (Pearson correlation coefficient  $r = -0.73$  to  $-0.83$ ) and  $H'_{CFF}$  ( $r = -0.54$  to  $-0.75$ ).

In multiple regression analysis, soil moisture content had a significant positive effect on number of CFF ( $p = 0.01$ ;  $r = 0.23$ ). Similarly, the total soil DNA amount was positively affected by soil moisture ( $p = 0.003$ ;  $r = 0.42$ ). The fungal DNA amount and *Trichoderma* spp. DNA amount were positively affected by soil moisture ( $p < 0.001$ ;  $r = 0.71$  and  $p = 0.01$ ;  $r = 0.69$ , respectively).

The mean air temperature on the sampling day (see Table 1) was significantly correlated with number of CFF ( $p = 0.006$ ,  $r = 0.35$ ) and fungal DNA proportion of total soil DNA amount ( $p = 0.03$ ,  $r = -0.65$ ) but not with total soil DNA ( $p = 0.02$ ; no correlation). A strong negative correlation was observed with fungal DNA amount ( $r = -0.81$ ) and with *Trichoderma* spp. DNA percentage of fungal DNA amount ( $r = 0.85$ ) but this was not confirmed in multiple regression analysis.

Land use intensity had a negative impact on  $H'_{CFF}$  ( $p = 0.004$ ,  $r = -0.61$ ), total soil DNA amount ( $p = 0.02$ ;  $r = 0.24$ ), fungal DNA amount ( $p < 0.001$ ;  $r = -0.85$ ), and a positive impact on the proportion of *Trichoderma* spp. DNA ( $p < 0.001$ ,  $r = 0.90$ ).

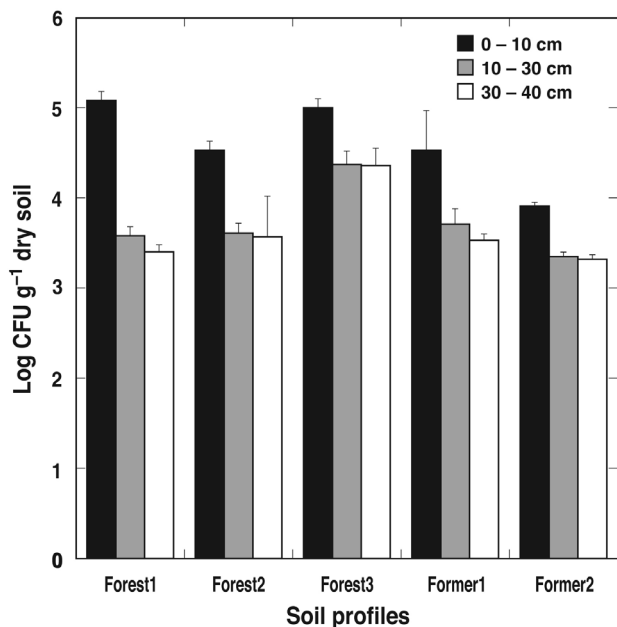


Fig. 5. The number of CFF in soil profiles in forests and former agricultural lands (mean  $\pm$  SD,  $n = 3$ ). The number of CFF was estimated on MEA after five days of incubation at  $20 \pm 2$  °C.

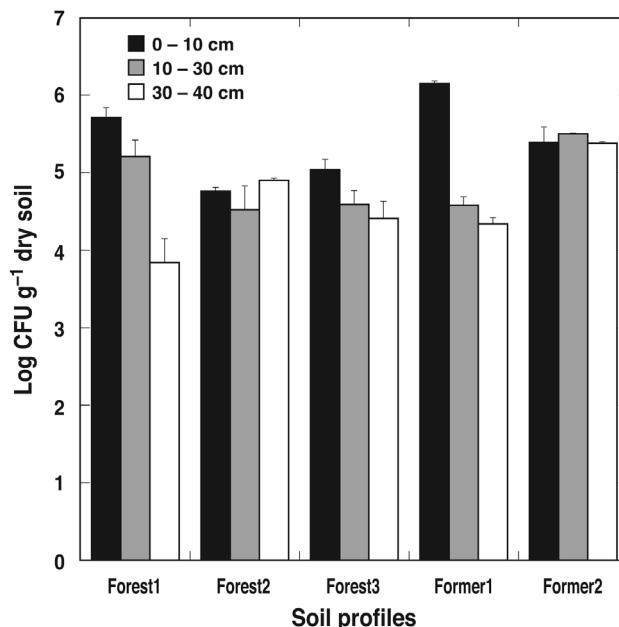


Fig. 6. Total number of cultivable microorganisms of soil profiles in forests and former agricultural lands (mean  $\pm$  SD,  $n = 3$ ). The number of cultivable microorganisms was estimated on MEA after five days of incubation at  $20 \pm 2$  °C.

Table 2. Predominant fungal genera and groups

Fungi	Forest1	Forest2	Forest3	Former1	Former2
Ascomycota					
<i>Penicillium</i>	✓	✓	✓	✓	✓
<i>Aspergillus</i>	✓	✓	✓	✓	
<i>Trichoderma</i>	✓	✓	✓		
<i>Verticillium</i>			✓		
<i>Acremonium</i>					✓
<i>Eladia saccula</i>			✓		
<i>Fusarium</i>				✓	
<i>Geomyces</i>			✓		
<i>Paecilomyces</i>					✓
<i>Spicaria</i>					✓
Zygomycota					
<i>Mucor</i>	✓	✓	✓	✓	✓
<i>Mortierella</i>	✓				
Sterile mycelia	✓	✓	✓	✓	✓

Multiple regression analysis showed that soil pH affected significantly only the number of CFF ( $p = 0.01$ ;  $r = -0.40$ ), but correlation analysis showed a positive correlation also with  $H'_{CFE}$  ( $r = -0.53$ ), and fungal DNA amount ( $r = -0.68$ ), and negative correlation with proportion of affected fungal DNA ( $r = -0.57$ ).

## Discussion

### Comparison of conventional and molecular methods

The correlation between the fungal diversity indexes

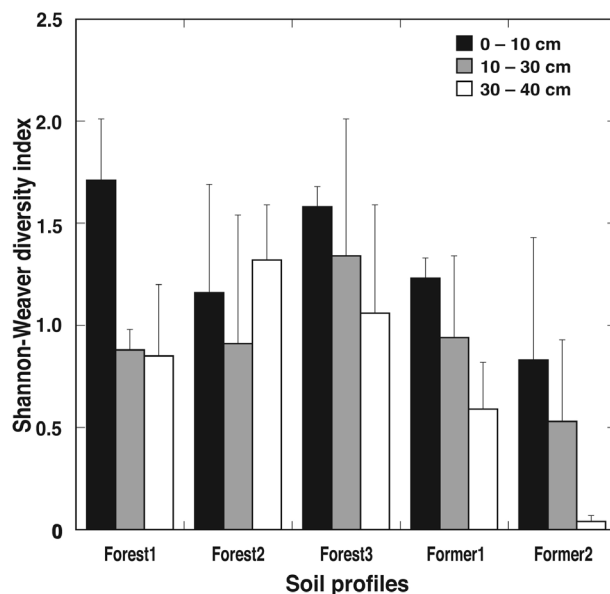


Fig. 7. Shannon-Weaver diversity index  $H'_{CFE}$  of soil profiles in forests and former agricultural lands (mean  $\pm$  SD,  $n = 3$ ).  $H'_{CFE}$  was calculated using CFU number of each CFF genus estimated on MEA after 10 days of incubation at  $20 \pm 2$  °C.

obtained by conventional plating methods ( $H'_{CFE}$ ) and by molecular methods ( $H'_{ARDRA}$ ) was weak ( $r = 0.45$ ). A stronger correlation of  $H'_{ARDRA}$  was found with number of CFF ( $r = 0.50 - 0.61$ ), total soil DNA amount ( $r = 0.54 - 0.72$ ), and fungal DNA amount ( $r = 0.58$ ). Values of  $H'_{ARDRA}$  were always higher than those of  $H'_{CFE}$ . The diversity estimated by conventional methods used data only on filamentous fungi

**Table 3.** The amount of fungal and *Trichoderma* spp. DNA in the upper soil layer (0 – 10 cm), n = 3

Profile	Total soil DNA ( $\mu\text{g g}^{-1}$ dry soil $\pm$ SD)	Fungal DNA [ $\text{ng g}^{-1}$ dry soil $\pm$ SD (% of total DNA)]	Number of rDNA copies $\text{g}^{-1}$ dry soil	<i>Trichoderma</i> spp. DNA [ $\text{ng g}^{-1}$ dry soil $\pm$ SD (% of fungal DNA)]	Number of <i>Trichoderma</i> spp. DNA copies $\text{g}^{-1}$ dry soil
Forest1	30.28 $\pm$ 13.78	1223.01 $\pm$ 247.06 (7.03)	$(3.24 \pm 0.65) \times 10^7$	10.27 $\pm$ 1.62 (0.79)	$(2.61 \pm 0.41) \times 10^5$
Forest2	4.17 $\pm$ 1.67	220.80 $\pm$ 22.22 (5.66)	$(5.84 \pm 0.59) \times 10^6$	1.57 $\pm$ 0.19 (0.64)	$(3.99 \pm 0.48) \times 10^4$
Forest3	9.98 $\pm$ 4.01	683.82 $\pm$ 116.44 (7.20)	$(1.81 \pm 0.31) \times 10^7$	2.33 $\pm$ 0.48 (1.01)	$(5.91 \pm 1.22) \times 10^4$
Average	14.81	709.21 (4.17)	$1.88 \times 10^7$	4.72 (0.81)	$1.20 \times 10^5$
Former1	8.09 $\pm$ 4.03	254.70 $\pm$ 39.15 (3.15)	$(6.74 \pm 1.04) \times 10^6$	2.14 $\pm$ 0.66 (0.84)	$(5.43 \pm 1.68) \times 10^4$
Former2	7.69 $\pm$ 5.00	260.84 $\pm$ 30.60 (3.39)	$(6.90 \pm 0.81) \times 10^6$	5.49 $\pm$ 0.33 (2.11)	$(1.39 \pm 0.08) \times 10^5$
Average	7.89	257.77 (3.27)	$6.82 \times 10^6$	3.82 (1.48)	$9.70 \times 10^4$
Meadow1	9.66 $\pm$ 3.61	136.00 $\pm$ 2.12 (1.41)	$(3.60 \pm 0.06) \times 10^6$	5.14 $\pm$ 0.26 (3.78)	$(1.30 \pm 0.07) \times 10^5$
Meadow2	12.18 $\pm$ 3.12	192.93 $\pm$ 7.46 (1.58)	$(5.11 \pm 0.20) \times 10^6$	5.55 $\pm$ 0.21 (2.87)	$(1.41 \pm 0.05) \times 10^5$
Meadow3	8.40 $\pm$ 6.18	106.68 $\pm$ 5.03 (1.27)	$(2.82 \pm 0.13) \times 10^6$	3.13 $\pm$ 0.63 (2.93)	$(7.94 \pm 1.60) \times 10^4$
Average	10.08	145.02 (1.42)	$3.84 \times 10^6$	4.61 (3.19)	$1.17 \times 10^5$
Field1	7.06 $\pm$ 0.47	103.44 $\pm$ 9.87 (1.47)	$(2.74 \pm 0.26) \times 10^6$	5.16 $\pm$ 0.40 (4.99)	$(1.31 \pm 0.10) \times 10^5$
Field2	5.27 $\pm$ 2.48	113.84 $\pm$ 23.83 (2.16)	$(3.01 \pm 0.63) \times 10^6$	3.05 $\pm$ 1.03 (2.68)	$(7.74 \pm 2.61) \times 10^4$
Field3	3.47 $\pm$ 4.28	84.47 $\pm$ 13.42 (2.43)	$(2.24 \pm 0.36) \times 10^6$	3.81 $\pm$ 0.16 (4.51)	$(9.67 \pm 0.41) \times 10^4$
Average	5.27	100.58 (2.02)	$2.66 \times 10^6$	4.01 (4.06)	$1.02 \times 10^5$

**Table 4.** Impact of different factors on the analyzed parameters. NS, not significant; NE, not estimated

	Number of CFF	Total number of microorganisms	$H'_{CFF}$	$H'_{ARDRA}$	Fungal DNA amount	<i>Trichoderma</i> spp. DNA amount
Sampling depth	p = 0.0001 (negative)	p = 0.01 (negative)	p = 0.007 (negative)	p = 0.005 (negative)	NE (negative)	NE (negative)
Soil moisture content	p = 0.01	NS	NS	NS	p < 0.001	p = 0.01
Soil pH	p = 0.01 (negative)	NS	NS	NS	NS	NS
Average air temperature of the sampling day	p = 0.006	NS	NS	NS	NS	NS
Land use type	NS	NS	p = 0.004 (negative)	NS	p < 0.001 (negative)	NS
Multiple R <sup>2</sup>	0.81	0.49	0.66	0.28	0.97	0.59
Probability of the model	p = 0.0003	p = 0.02	p = 0.001	p = 0.01	p < 0.001	p = 0.03

and excluded yeasts. In molecular analysis the diversity of yeast genomes also contributed to the estimated diversity, since they are amplified with primers ITS4 and ITS1F alongside with other representatives of *Ascomycota* or *Basidiomycota*. Further, conventional methods can identify cultivable microorganisms, while the  $H'_{ARDRA}$  index reveals the diversity “of some of the most abundant community members” (Kowalchuk et al. 2006). In other investigations the detection threshold of ARDRA gels is quite high, and can detect the DNA of two different strains in various proportions at ratios as low as 1/20 (Grundmann, Normand 2000).

In most soil profiles the highest  $H'_{ARDRA}$  and  $H'_{CFF}$  index was found in the upper soil layers. However, in two soil profiles (Forest2 and Field2), the highest diversity  $H'_{ARDRA}$  was found at a depth of 10 – 30 cm, and in the Forest2 soil profile the richest community of  $H'_{CFF}$  was in the deepest soil layer (30 – 40 cm). These differences were probably not caused by soil moisture or soil pH, but can be explained by the history of soil profile development (see further).

In profile Forest3, the estimated fungal diversity was significantly higher than in the other two forest profiles, which might be caused by different soil types (Haplic Arenosols in Forest3 compared to Haplic Cambisols in the



other two forest profiles) and/or with the associated forest vegetation type.

In North Carolina in two forest sites with loblolly pine (*Pinus taeda*) and mixed hardwood the highest fungal richness was in the O horizon (organic material and detritus), intermediate values were obtained in horizons L (litter) and A (organic matter-rich mineral soil), and the lowest in the B horizon (clay-rich mineral soil) (O'Brien et al. 2005). The authors found that in general the diversity and richness was correlated positively with clone library size.

The highest diversity of cultivable microfungi and yeasts in Austria was found in the top layer of the forest soil (0 – 15 cm) without temporal flooding (Wuczkowski et al. 2003). This layer of the soil profile was characterized as a zone of the highest degradation of the organic material. These results are similar to ours with the exception of the soil profile Forest2. In agricultural soils with conventional farming the highest diversity was recorded at a depth of 30 – 35 cm caused by agricultural practices (Wuczkowski et al. 2003). In our investigation the  $H'_{ARDRA}$  diversity in agricultural soil at a depth of 10 – 30 cm was similar or slightly higher than that in the upper soil layer. In the case of the profile Field2, the diversity was lower in deeper soil (depth of 30 – 40 cm). Since this soil layer was not analyzed in the other two fields, it is difficult to make any general conclusions about the fungal diversity in this depth.

In soils of Sweden, the fungal species richness investigated using terminal-restriction fragment length polymorphism was significantly higher in newly abandoned agricultural land than in actively used agricultural fields and forest (Klamer, Hedlund 2004). In our investigation former agricultural soils had the same diversity  $H'_{ARDRA}$  as other soil groups. The set-aside land in Sweden had not been used for five years and had not reached an equilibrium state with respect to competition between species, and thus represented higher diversity than soil of a forest, which represents a climax community. The abandoned agricultural lands studied in Latvia had not been used approximately for 15 years and thus the microbial community had progressed substantially toward a climax status.

The structure of dominant DNA fragments and consequently of the communities change at different soil depths, as shown in gel A (Fig. 3). This tendency was also observed regarding CFF; for example, in forest soils the amount of sterile mycelia proportionally increased with increasing depth. O'Brien et al. (2005) observed that mycorrhizal species predominate deeper in the soil profile whereas saprophytic species predominate in the litter layer. Further sequencing data will demonstrate if the sterile mycelia in our soil profiles was formed by mycorrhizal species.

The correlation between the number of CFU of *Trichoderma* spp. and *Trichoderma* spp. DNA amount was weak ( $r = 0.20$ ). This can be explained by the fact that not

all species or strains of *Trichoderma* genus can sporulate under standard laboratory conditions (Ellison et al. 1981; Schrüfer, Lysek 1990) and they may have been counted as sterile mycelia.

#### *Distribution of cultivable microorganisms*

The number of CFF and the total number of cultivable microorganisms tend to decrease with soil depth. An exception was soil profile Forest2 in which the highest number of cultivable microorganisms was at a depth of 30 – 40 cm, and soil profile Former2 in which the number of cultivable microorganisms was similar at all depths. This can be explained by the past history of profile development. In the profile Forest2 the upper 30 cm layer was composed of younger colluvial material with the original profile starting at 30 cm. The soil at Former2 was previously ploughed which destroyed the original stratification of the microorganisms.

A decrease in amounts of microorganisms in deeper soil horizons has been observed in investigations using different methods – phospholipid fatty acid analysis (Fierer et al. 2003; Fritze et al. 2000), ergosterol level (Krivtsov et al. 2007), and denaturing gradient gel electrophoresis (Krave et al. 2002). Nevertheless, some exceptions have also been described. For example, a bacterial peak has been observed at a 42.5-cm depth in the peat profile of a spruce (*Picea abies*) and birch (*Betula pubescens*) forest in Denmark (Ekelund et al. 2001) caused by partial anaerobic conditions, higher water content and higher organic matter content deeper in the soil. An even distribution of microorganisms without a decrease in their number with depth was found in a cryogenic weakly solidized loamy sandy pale soil of Yakutia (Ivanova et al. 2008). There is no permafrost in the subsoil of Latvia, but cryoturbation of the soil during winter may have some effect.

The CFF identified in our work are representatives of genera that have been isolated from a broad range of soils. For example, in the investigation of Wuczkowski et al. (2003), in forest soils of *Salix* and *Populus* stands and conventional agriculture soils the most abundant fungal genera were *Acremonium*, *Cladosporium*, *Penicillium*, *Cylindrocarpon* and *Trichoderma*. In boreal forest stands of *Picea mariana*, genera *Umbeopsis*, *Mortierella* and *Penicillium* were reported to be common (Summerbell 2005). In our work the most abundant genera were *Penicillium* and *Mucor* (in all analyzed soil profiles) as well as *Aspergillus* and *Trichoderma*, in some soil profiles also *Mortierella* and *Acremonium* were identified.

#### *Quantification of fungal DNA*

According to the qPCR results, fungal DNA contributed only 1.13 – 5.51% of the total extracted soil DNA (Table 3) which was several times lower amount than that found in North-Western France (Gangneux et al. 2011).

In forest lands and former agricultural lands with natural

afforestation the proportion of fungal DNA within the total soil DNA was significantly higher than in agricultural land types – 2.21 – 5.51% in forests and abandoned agricultural lands compared to 1.13 – 2.17% in currently used agricultural lands. Interestingly, the higher amount of total soil DNA of Forest1 (Fig. 1) in the upper soil horizon was not associated with a different population of fungal DNA compared to that in other forest soil samples. A strong correlation between fungal DNA amount and the period since last tillage was shown in an investigation carried out in North-Western France – the lowest fungal DNA amount was observed in fields with conventionally cultivated crops, and the highest in grasslands (Gangneux et al. 2011).

The amount of *Trichoderma* spp. DNA varied from 0.64 to 4.99 % of the total fungal DNA, and the highest relative abundance of *Trichoderma* spp. DNA was observed in agricultural soils.

Multiple linear regression analysis results (Table 4) showed that increased soil moisture content resulted in higher amounts of fungal DNA and *Trichoderma* spp. DNA. The amount of fungal DNA was affected also by land use type.

Higher fungal-bacterial DNA ratios have been previously observed in forest soil in comparison to pasture and cultivated soil (Lauber et al. 2008). In beech forest soil (Dystic Cambisol) in southern Germany the mean number of *Trichoderma* spp. ITS region copies was lower ( $1.2 \times 10^6$  g<sup>-1</sup> of soil fresh mass) than in arable soil (Loamy Cambisol) –  $1.3 \times 10^6$  g<sup>-1</sup> of soil fresh mass (Hagn et al. 2003). Considering that the ITS region is amplified in fungal genome in even several 100 copies (Simon, Weiß 2008), our calculation of *Trichoderma* genome copies (Table 3) was in the same range as the data obtained from soils in Germany.

#### *Impact of different factors on the analyzed soil microorganisms*

Multiple regression analysis showed that the number of CFF was negatively affected by increasing soil pH. In medium acid soils (forest soils – pH 4.46 – 5.30), fungal abundance was higher in comparison with neutral soils (all other soil groups – pH 6.68 – 7.90), since fungi prefer more acidic environments than bacteria. The impact of soil pH on soil microbial communities has been widely described previously. In boreal forests fungal biomass is very high in acid soil (pH < 4.1) (Högberg et al. 2007).

Soil moisture content in general has a positive impact on the number of CFF, fungal DNA amount and *Trichoderma* spp. DNA amount. Similarly, increasing moisture content was shown to cause higher biomass and *H'* index of soil fungi communities in Zoige Alpine Wetland (Feng et al. 2009).

Multiple regression showed that air temperature can have some effects on soil microbial populations. Study of the seasonality of microbial population dynamics has shown that soil microbial communities undergo a shift in

function and genetic structure between winter and summer (Lipson et al. 2002).

Land use type affected the diversity of CFF, total soil DNA, fungal DNA amount and the proportion of *Trichoderma* spp. DNA. In a similar investigation in the southeastern United States in which hardwood forests, pine forests, cultivated and livestock pasture lands were compared, it was determined that the composition of fungal communities was most strongly correlated with specific soil properties (soil nutrient status) rather than land use types (Lauber et al. 2008).

We found no significant differences between fungal communities from meadows and arable lands – the diversity  $H'_{ARDRA}$  values were similar, while the proportion of fungal DNA and proportion of *Trichoderma* spp. DNA were slightly higher (not significant) in arable lands. Similarly, no substantial differences in *H'* indexes of arbuscular mycorrhizal fungi between meadows and arable lands in Central Europe were found by Oehl et al. (2003) but using other methods significant differences between soil groups were shown.

Conventional microbiological and molecular biology methods for investigating soil fungal communities showed similar results regarding relationships between depth and moisture and abundance of fungi. The numbers of CFF and the total number of cultivable microorganisms tended to decrease with increasing depth, which is in line with previous studies. There was a weak correlation between  $H'_{ARDRA}$  and  $H'_{CFF}$  diversity indexes, and the values were higher for  $H'_{ARDRA}$ . Both approaches showed higher fungal diversity at depths of 0 – 10 cm and 10 – 30 cm in comparison with a depth of 30 – 40 cm. The proportion of fungal DNA within the total soil DNA was significantly higher in forests and abandoned lands than in agricultural lands. The amount of *Trichoderma* spp. DNA was similar in all soil groups, but its proportion of the total fungal DNA amount was higher in meadows and arable lands.

An increase of land use intensity had a negative effect on the diversity of cultivable filamentous fungi (comparing forest lands with abandoned agricultural lands) and on the fungal DNA amount (comparing arable lands and meadows with forest lands and abandoned agricultural lands).

Our analysis showed that different soil types in Latvia show common features of distribution of fungal organisms, similar to the pattern found in other climatic zones and soil types. Natural phenomena (floods) and tillage causes changes in the distribution of soil fungal populations, which remain for many years. We demonstrated that molecular and conventional methods of analysis show rather weak correlation in determining total amount of soil fungi and their distribution among soil horizons. Both approaches showed the impact of soil moisture, land use type and air temperature on fungal communities. Correlation between fungal species diversity indices obtained by molecular and conventional plating methods was low. Molecular methods

gave higher diversity values, but more evenly distributed diversity of fungal species in different soil types and horizons.

The comparative analysis of fungal communities in soils under different types of natural forests, actively used and abandoned agricultural lands has provided baseline information about the fungal diversity and composition in these ecosystems although the obtained results are very variable due to the fact that each soil profile was unique in terms of soil type and land use.

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