

Monitoring seasonal changes in microbial populations of spruce forest soil of the Northern Temperate Zone

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Abstract. Soil microbial populations in the Northern Temperate Zone have been poorly studied in comparison with extreme environments. The aim of the work was to study the seasonal changes in the microbial populations of spruce forest soil of the Northern Temperate Zone using classical methods of microbiology and molecular biology. Upper horizons in two *Picea abies* stands on sod-podzolic and illuvial humus podzol soil were analysed. Sampling was done monthly over a period of twelve months (May 2009–April 2010). Microbial communities in both experimental plots showed different responses to the analysed environmental factors. In the sod-podzolic soil only the fungal DNA amount was significantly higher in the rest period (October–April) in comparison with the active vegetation period (May–September) and the number of *Penicillium* spp. colonies was larger in the active vegetation period. In the other soil the number of maltose utilizing bacteria, yeasts, and *Penicillium* spp. and other culturable filamentous fungi was significantly higher in the active vegetation period, while the fungal DNA amount was elevated in the rest period. Although ARDRA did not reveal differences, sequencing of 84 fungal isolates showed different compositions of the communities. Sørensen's index between the plots was low (0.29). Comparing the active vegetation period with the rest period, the index was higher (0.48). Although all tested fungal isolates from the rest period were able to grow at 4°C, none of them showed psychrotrophic growth characters.

Key words: forest soil, filamentous fungi, active vegetation period, rest period, Shannon–Weaver diversity index, qPCR.

INTRODUCTION

Several studies are available about the impact of seasonal changes upon soil microorganisms in arctic soils (Nemergut et al., 2005), deserts, tropical soils, and other soils in extreme environments (Dion, 2008). Comparatively few investigations have been published about microbial populations in the soils of the Northern Temperate Zone (NTZ) in which climatic conditions during winter are charac-

terized by permanent or temporal snow cover and a significant decrease of topsoil temperature below zero.

Functional diversity of bacterial populations was assessed in sandy soils over a climatic gradient in Western Canada using sole-carbon-source utilization analyses (Staddon et al., 1998). Response of soil bacteria and endomycorrhizal fungi to nitrogen deposition in northern forest ecosystems was investigated in north-western Lower Michigan (Waldrop et al., 2004). Studies on the seasonal dynamics of the microbial population of alpine dry meadow sites in the Colorado Rocky Mountains revealed that the microbial community undergoes a shift in function and genetic structure at the change of winter and summer seasons (Lipson et al., 2002). Microbial biomass and activity increase to maximal levels in late winter under the snow and then decline during snowmelt. This results in an increased availability of nitrogen, which is used by plants and the developing summer microbial community (Brooks et al., 1998; Lipson et al., 1999, 2000; Schmidt et al., 2004). Potential allelochemicals, such as simple phenolic compounds, are released from fresh litter in autumn and make up carbon and energy sources for the microbial community that develops in autumn and winter (Lipson et al., 2000, 2002). These potential allelochemicals are consumed before plant growth starts in spring (Schmidt & Lipson, 2004). In NTZ ecosystems litter decomposition (Zimov et al., 1993; Schmidt & Lipson, 2004) and gas accumulation (e.g., CO₂, CH₄, N₂O) take place under the winter snow packs. In the soil of jack pine (*Pinus banksiana*) stands in Western Canada Staddon et al. (1998) found significant positive correlations between the diversity of microbial populations and the soil pH, average daily temperature in July, mean annual temperature, degree-days above 18°C, and degree-days above 5°C.

The aim of our work was to analyse seasonal changes in the microbial populations in NTZ spruce (*Picea abies*) forest soil from two closely located forest stands with the same dominant tree species, but with different relief, soil, and vegetation types using classical methods of microbiology and molecular biology. The results confirmed that microbial populations are active under the snow during winter, while the two analysed soils showed different responses to environmental factors.

MATERIALS AND METHODS

Experimental plots

Two 40-years-old Norway spruce (*Picea abies* (L.) Karst.) stands in Malpils municipality (ca 30 km east of Riga, Latvia) were analysed. The plots were located ca 200 m from each other. Soil1 was sod-podzolic soil (Cutanic, Stagnic Albeluvisols) with *Oxalidosa* forest type. Soil2 was illuvial humus podzol (Placic, Rustic, Albic, Follic, Stagnic Podzols) with *Myrtilloso-polytrichosa* forest type (forest type classification according to Buss (1997), and soil types according to Latvian and FAO WRB (2006) soil classifications). Forest stands were heavily infected with the root rot fungus *Heterobasidion parviporum* (Grantina et al., 2010).

Soil1 was located at the upper part of a hill on moraine sediments and Soil2 in the depression of the relief on glaciofluvial sediments. The topography caused differences in the soil drainage between the experimental plots; the groundwater level was higher in Soil2. During winter only upper horizons of Soil1 were frozen (I–III). The snow cover of ca 0.50 m thickness lasted from January until mid-March.

Meteorological data

The raw meteorological data were obtained from the database of the Latvian Environment, Geology and Meteorology Centre, as recorded at the Meteorological Station in Riga. Average daily and monthly air temperature and total precipitation for every month were calculated.

Soil sampling and physico-chemical analysis

Soil samples for the microbiological analysis were taken in the middle of every month (9th–12th day, May 2009–April 2010) from 0–10 cm and 11–30 cm depths. Observations were divided into active vegetation period (AVP; May–September, average daily temperature $t > 10^{\circ}\text{C}$) and rest period (RP; October–April, $t < 10^{\circ}\text{C}$). A soil sampling plot was a circle with three transects on which nine samples (100 g) were taken at both depths at distances of 0.3 m, 1.0 m, and 3.0 m from the centre. Samples from the same depth were combined, resulting in three samples from both depths. Samples were placed in sterile plastic bags (*Nasco* WHIRL-PAK), stored at $+4^{\circ}\text{C}$ for a few days until the plate count of culturable microorganisms was made, and after that stored at -20°C . These samples were used also for the estimation of soil moisture content according to ISO 11465. For all other physico-chemical analyses soil samples (1–3 kg) were taken from each soil horizon in September 2009. Chemical analyses were made using (Expert Panel on Soil, 2006).

Analysis of culturable soil microorganisms

In order to estimate the number of colony forming units (CFU) of culturable filamentous fungi (CFF), yeasts, and maltose utilizing bacteria by a plate count method, soil sample dilutions were prepared by adding 10 g of soil to 90 mL of sterile distilled water. Suspensions were homogenized for 1 h on a horizontal shaker. After that serial dilutions were prepared, and 0.1 mL of dilutions 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5} were used. Agarised malt extract (MEA; 30 g/L, pH 5.5, from Biolife) was used as a growing medium that is favourable for fungi, yeasts, and certain groups of bacteria. Further in the text all these groups together are referred to as culturable microorganisms. The incubation time was 5 days and the temperature was $20 \pm 2^{\circ}\text{C}$. The CFU were expressed per gram of dry soil. Every soil sample was analysed in three replicates.

Genera of filamentous fungi were determined after 10 days of incubation according to morphological characteristics and light microscopy results.

Extraction of total soil DNA and quality control

Total soil DNA was extracted with a PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc.), which uses harsh lysis for DNA extraction. Samples (250 mg) were homogenized using a horizontal Mixer Mill Type MM 301 (Retsch) at a maximal speed of 30 Hz (1800 oscillations/minute) for 10 min. The amount and purity of the DNA were established spectrophotometrically using Ultrospec 3100 Pro (Amersham Biosciences) at wavelengths of 230, 260, and 280 nm in order to determine the contamination with proteins and humic compounds (Yeates et al., 1998).

ARDRA of soil DNA

For the amplified ribosomal RNA gene analysis (ARDRA) the fungal rRNA gene region that contains two internal transcribed spacers (ITS) and the 5.8S rRNA gene (ITS1-5.8S-ITS2) was amplified with the primers ITS1F and ITS4 (Gardes & Bruns, 1993).

The reactions in Eppendorf Mastercycler Personal were carried out in 50 µL volume. The mixture contained 0.4 µL of Hot Start *Taq* DNA Polymerase; 5 µL of 10X Hot Start PCR Buffer; 5 µL of dNTP Mix, 2 mM each; 4 µL of 25 mM MgCl₂; 0.75 µL of Bovine Serum Albumin 20 mg/mL (all reagents from Fermentas); 1 µL of each 25 µM primer (OPERON Biotechnologies); 30.85 µL of sterile distilled water; and 1 µL of DNA template. The polymerase chain reaction (PCR) conditions were as follows: the initial denaturation step of 4 min at 95°C, 40 s of denaturation at 95°C, 40 s of annealing at 52°C, 1 min of primer extension at 72°C (30 cycles), and final extension 10 min at 72°C.

For ARDRA analysis with restriction endonuclease *Bsu*RI (Chabrierie et al., 2003) the amplification products after the PCR were precipitated by 450 µL of 90% ethanol and 0.3 M sodium acetate (pH 5.0). The precipitated DNA was washed with 70% ethanol, air dried, dissolved in ddH₂O, and digested with *Bsu*RI (Fermentas). The restriction products were visualized in 6% polyacrylamide gel electrophoresis using a Mighty Small™ II (Hoefer) unit. Gels were photographed and analysed with KODAK1D software. For the calculation of the Shannon–Weaver diversity index (H') the following equation was used: $H' = -\sum p_j \log_2 p_j$, where p_j = relative intensity of individual band (Gabor et al., 2003).

Quantitative PCR

In quantitative PCR (qPCR) the ITS1-5.8S-ITS2 rRNA gene region of higher fungi with primers ITS1F and ITS4 was used to determine the amount of total fungal DNA within the total soil DNA. Combined soil DNA samples for each soil depth were analysed in three replicates for every month's samples.

As both forest stands were infected with *H. parviporum*, we monitored with special interest the DNA amount of antagonistic fungi *Trichoderma* spp. of this tree pathogen (Korhonen & Stenlid, 1998). In order to estimate the amount of

Trichoderma spp. DNA, qPCR with the primers uTr and uTf (Hagn et al., 2007) was performed. Samples of every second month were used, each analysed in three replicates.

The reactions were carried out in 25 µL volume. The mixture contained 12.5 µL of Maxima™ SYBR Green qPCR Master Mix 2x (Fermentas), 1 µL of each 25 µM primer, 9.5 µL of sterile distilled water, and 1 µL of the DNA template. The PCR conditions on 7300 Real time PCR system (Applied Biosystems) were as follows: initial denaturation 60 s at 95°C; 40× (30 s of denaturation at 95°C, 30 s of annealing at 55°C for primers ITS1F and ITS4 or 60°C for primers uTr and uTf, 30 s of primer extension at 72°C). Calibration curves were built using serial dilutions of DNA from pure cultures of *Phlebiopsis gigantea* MSCL 702 and *Trichoderma harzianum* MSCL 309. In more detail the method is described in (Grantina et al., 2011).

Sequencing

A total of 84 isolates (45 sporulating, 39 sterile mycelia, i.e. not sporulating when kept at +4°C for several months) representing dominant filamentous fungi were isolated from the plates used for the enumeration of CFF and subcultured on MEA. Genomic DNA from approximately 0.25 g of mycelia was extracted using the method developed by Cenis (1992). The extracted DNA was amplified in PCR with the primers ITS4 and ITS1F. Afterwards 5 µL of amplified products was subjected to a sequencing protocol with BigDye Terminator v. 3.1 Cycle Sequencing Kit with primer ITS1F. The sequencing of the samples was performed in the Latvian Biomedical Research and Study Center. The obtained sequences were analysed using Staden Package 1.6.0. and checked against the database of the National Centre for Biotechnology Information using the Basic Local Alignment Search Tool for identification.

From the data of the sequenced isolates Sørensen's community similarity index C_s was calculated using the following equation: $C_s = 2C/A + B$, in which A and B are the number of species in samples A and B (Soil1 and Soil2), respectively, and C is the number of species shared by the two samples (Izzo et al., 2006).

Screening for psychrotrophic and mesophilic fungi

For growth temperature tests 29 strains (19 from RP, 10 from AVP) were used. Colonies were incubated at $4 \pm 2^\circ\text{C}$ and at $20 \pm 2^\circ\text{C}$ on MEA. Growth (expressed as colony diameter) was assessed several times during 20 days and growth rate (expressed as mm/day) was calculated.

Statistical analysis

The *F*-test, *t*-test ($\alpha = 0.05$), and correlation analysis were made with Excel (Microsoft, USA). Multiple regression analysis of the results and cluster analysis of ARDRA fingerprints were performed with the R package (R Development Core Team, 2009).

RESULTS AND DISCUSSION

Results of the meteorological data analysis

The average air temperature and total monthly precipitation during the analysed period are given in Fig. 1. The highest average air temperature at the meteorological station in Riga was recorded in June (+16.68 °C), the lowest in January (−10.41 °C). The smallest amount of precipitation was recorded in May (9.70 mm) and the highest in June (136.80 mm).

Soil characterization

Data on the element content and total nitrogen content in each horizon of both soil profiles are listed in Table 1. Information about soil texture is given in Table 2. Certain differences in the element content between the profiles are caused by soil formation processes and later influence of geological sediments and topography: moraine contains more exchange cations; nitrogen accumulates in the lower parts of the relief.

In either experimental plot there were no statistically significant differences of soil moisture between sampling depths (Fig. 2). However, due to the landscape

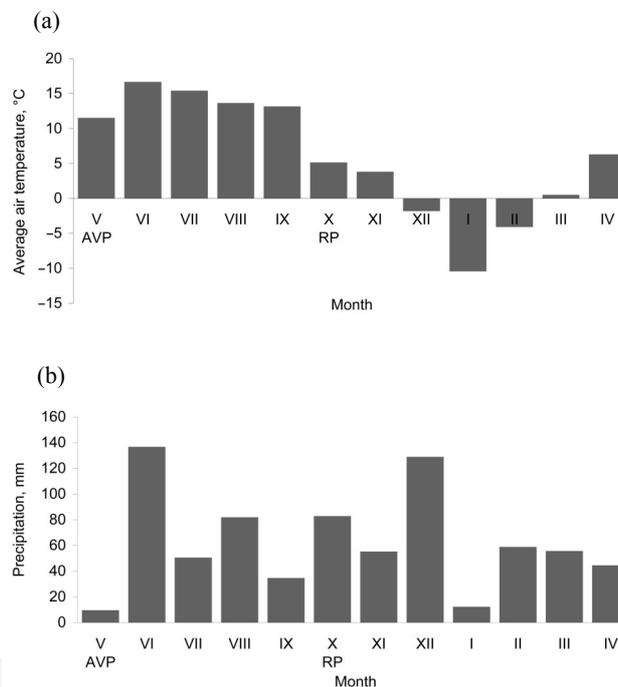


Fig. 1. Average air temperature (a) and total monthly precipitation (b) in the observation station at Riga during the analysed period. For the calculations raw meteorological data obtained from the database of the Latvian Environment, Geology and Meteorology Centre were used.

Table 1. Element content by soil horizons in the experimental plots

Horizon	Depth, cm	Ca, mg kg ⁻¹	Mg, mg kg ⁻¹	K, mg kg ⁻¹	Na, mg kg ⁻¹	P, mg kg ⁻¹	N, %	pH _{KCl}
Soil1								
O	0–2	824.67±109.86	334.40±15.95	618.00±351.64	60.00±3.46	142.32±1.16	0.63±0.00	3.22±0.02
Ah	3–19	70.07±13.58	272.93±25.00	814.00±365.46	80.00±15.10	146.51±1.54	0.11±0.00	3.68±0.02
B	20–35	109.73±13.67	213.40±12.39	1140.67±49.57	70.00±10.00	103.13±1.34	0.06±0.00	3.98±0.01
EB	36–45	462.07±110.72	273.33±54.79	1318.00±3.46	98.00±10.58	63.76±0.77	0.06±0.00	3.56±0.01
Bt 1	46–81	2664.00±270.25	338.67±137.29	1320.00±3.46	140.67±51.47	76.69±0.59	0.00	3.95±0.00
Bt 2	82–130	2696.67±9.02	292.80±24.31	1282.00±10.39	125.33±23.44	125.80±1.32	0.00	4.62±0.02
Soil2								
O	0–11	2262.00±326.56	49.60±3.41	846.67±371.07	86.00±12.49	306.04±0.78	0.37±0.32	2.52±0.02
AhE	12–22	355.47±13.0	7.47±3.78	196.00±27.06	106.00±34.00	241.65±1.32	0.31±0.00	2.42±0.02
E	23–30	76.40±21.54	15.93±12.47	100.00±16.00	81.33±8.33	24.99±0.45	0.06±0.00	3.42±0.01
Bs 1	31–45	132.87±16.15	78.20±9.89	178.67±15.01	82.00±13.11	49.66±0.59	0.06±0.00	3.73±0.01
Bsg	46–61	223.53±53.53	247.07±33.62	182.00±7.21	71.33±11.02	72.09±0.97	0.06±0.00	3.88±0.01
Bs 2	62–102	310.60±4.85	230.80±17.17	200.00±19.29	76.00±16.37	82.96±0.77	0.00	4.04±0.02
R	103–135	409.47±100.82	252.73±30.66	249.33±36.90	54.00±3.46	111.29±1.38	0.00	4.08±0.01

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Table 2. Soil granulometry by soil horizons in the experimental plots

Horizon	Clay, %	Silt, %	Sand, %	Characterization
Soil1				
O	Organic horizon			
Ah	10.9	24.7	64.4	Sandy loam
B	11.5	25.9	62.6	Sandy loam
EB	19.1	25.3	55.6	Sandy loam
Bt 1	22.5	26.7	50.8	Sandy clay loam
Bt 2	20.4	29.6	50	Loam
Soil2				
O	Organic horizon			
AhE	8.9	15.5	75.6	Loamy sand
E	3.8	5.2	91	Sand
Bs 1	6.2	2.8	90	Sand
Bsg	4.9	2.5	92.6	Sand
Bs 2	5.1	1.5	93.4	Sand
R	4.2	2.6	93.2	Sand

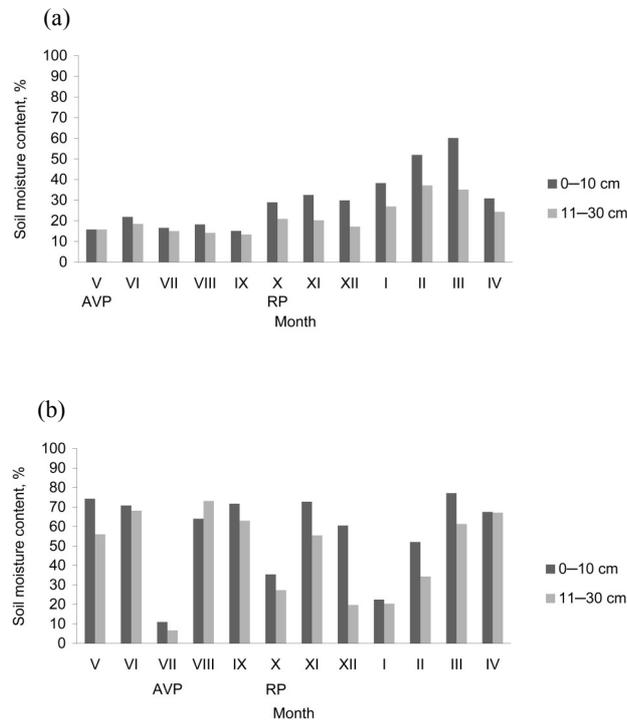


Fig. 2. Changes in soil moisture content during the analysed period in sampling plots Soil1 (a) and Soil2 (b) at two sampling depths ($n = 1$). Composite soil samples from each depth were used to estimate soil moisture content.

topography the moisture content was on average significantly higher in Soil2 ($F = 11.308$; $p < 0.001$). The topography of the plot and the presence of sphagnum moss caused ample fluctuations in its moisture content (6.69–72.55%). Still, in general there were no statistically significant differences of moisture content in Soil2 between the AVP and RP whereas in Soil1 the moisture content was significantly higher during the RP in comparison with the AVP ($F = 22.538$; $p < 0.001$).

The soil types and forest types of both analysed experimental plots are common in Latvia and in the NTZ. *Oxalidos* and *Myrtilloso-polytrichosa* forests cover 19.5% and 3.6%, respectively, of the whole forest territory of Latvia. Sod-podzolic soil is the most common soil type in Latvia, but illuvial humus podzol is common only in lowlands (Karklins et al., 2009).

Soil chemical analysis showed that the soils of both experimental plots were very poor in nitrogen (0.63% in Soil1 and 0.37% in Soil2) in comparison with average values for the same forest types (1.8% in *Oxalidos* forests and 1.5% in *Myrtilloso-polytrichosa* forests) in Latvia (Bardule et al., 2009).

The texture of Soil1 was typical of *Oxalidos* forests, while Soil2 had a decreased content of clay and silt, but an increased content of sand in comparison with average soils of the *Myrtilloso-polytrichosa* forests in Latvia (Bardule et al., 2009).

Results of analysis of culturable microorganisms

Changes in the number of CFF in the experimental plots during the investigated period are presented in Fig. 3a, b. In Soil1 the numbers of obtained fungal colonies did not differ significantly between the AVP and RP and the colonies were equally distributed at both sampling depths (on average $(4.70 \pm 3.95) \times 10^4$ CFU g⁻¹ dry soil). In Soil2 significant differences were observed between the two sampling depths ($F = 6.049$; $p < 0.05$) and between the AVP and RP ($F = 2.883$; $p < 0.05$; 2.15×10^5 and 1.84×10^4 CFU g⁻¹ dry soil, respectively), accompanied by a remarkable variability within the 12 months; for example, low numbers of CFF were detected in July, October, January, and February.

The numbers of CFU of yeasts and maltose utilizing bacteria are shown in Fig. 3c, d. In Soil1 they were significantly different between the two sampling depths in the AVP ($F = 1.150$; $p = 0.020$; $(1.32 \pm 0.41) \times 10^6$ CFU g⁻¹ dry soil at 0–10 cm depth, $(6.65 \pm 4.38) \times 10^5$ CFU g⁻¹ dry soil at 11–30 cm depth), but similar at both depths in the RP. In general, differences in CFU numbers in Soil1 between the AVP and RP were not significant. In Soil2 the numbers of this group of soil microorganisms were not statistically significantly different between the two sampling depths but differed significantly ($F = 68.048$; $p = 0.032$) between the AVP and RP, being $(2.28 \pm 1.88) \times 10^6$ CFU g⁻¹ dry soil on average and $(3.62 \pm 3.51) \times 10^5$ CFU g⁻¹ dry soil, respectively.

So on average bacterial and fungal populations were in higher numbers in the AVP only in Soil2. Higher microscopic counts of bacteria in summer were recorded also by Lipson et al. (2002) in alpine dry meadow soils, but fungal

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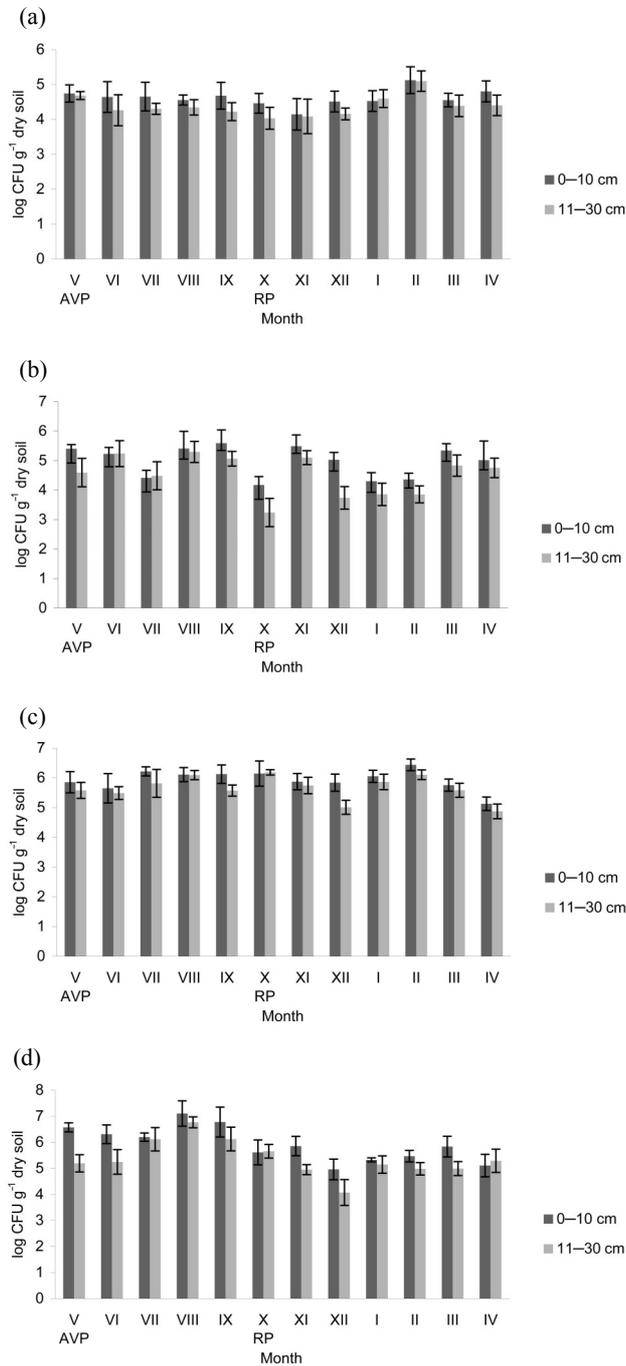


Fig. 3. Changes in the log number of CFF (a, b) and yeasts and maltose utilizing bacteria (c, d) during the investigated period in sampling plots Soil1 (a, c) and Soil2 (b, d) at two sampling depths (\pm SD; $n = 9$). Results were obtained after five days of incubation on MEA at the temperature of $20 \pm 2^\circ\text{C}$.

abundance expressed as length of hyphae (m g^{-1} soil) was increased in winter. These soils differ from our experimental plots in terms of vegetation and soil type, and they have at least two times higher nitrogen content in the A horizon (7.4 g N kg^{-1}). The authors concluded that winter microbial communities utilize more complex substrates such as cellulose but to a lesser extent simple compounds than do the summer communities.

As to particular dominant CFF genera on MEA, the percentage of *Penicillium* spp. was on average significantly higher during the AVP than during the RP in both plots: 33.20% versus 16.52% ($F = 2.407$; $p = 0.010$) in Soil1 and 38.36% versus 21.42% in Soil2 ($F = 2.415$; $p = 0.016$) (Fig. 4). Certain species such as *P. canescens* (one isolate) and *P. montanense* (four isolates) were detected only in the AVP, isolates of *P. spinulosum* (four isolates in total) were obtained in both periods according to sequencing results (Table 3).

Members of the genus *Mortierella* were evenly abundant in both the AVP and the RP in Soil1: 9.16% and 8.38%, respectively. In Soil2 the percentage of *Mortierella* spp. was low in the period July–January (on average 3.12%), but high in the periods May–June (29.93%) and February–April (24.71%). Looking at the particular species according to sequencing results, isolates of *M. alpina* (one isolate) and *M. gamsii* (one isolate) were detected only in the RP and *M. humilis* (one isolate) in the AVP, but isolates of *M. macrocystis* (four isolates in total) were detected in both periods.

The percentage of *Trichoderma* spp. was on average low in both plots, and at several sampling times *Trichoderma* spp. were not detected at all, e.g., in May–July and November–December in Soil1, and in October–November in Soil2. Detailed identification of fungal isolates using rRNA gene sequencing revealed that *Hypocrea viridescens* (March, one isolate) and *T. asperellum* (February, two isolates) were recovered only in the RP, but *H. pachybasioides* (six isolates in total) and *T. viride* (eight isolates in total) occurred both in the RP and AVP.

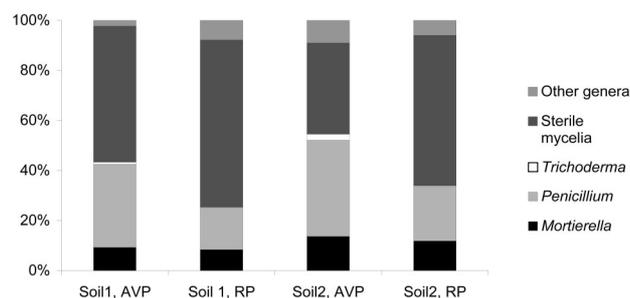


Fig. 4. Average percentage of the dominant CFF genera in the AVP and RP in the two sampling plots. Other genera include members from such genera as *Aspergillus*, *Botrytis*, *Cladosporium*, *Geomyces*, *Gilmaniella*, and *Mucor*. Results were obtained after ten days of incubation on malt extract agar at the temperature of $20 \pm 2^\circ\text{C}$.

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Table 3. Fungal isolates of Soil1 and Soil2 identified by sequencing

Isolate ^a	CFU g ⁻¹ dry soil	Homolog sequence in NCBI	Max identity, %	Strain in MSCL ^b
Soil1, AVP				
<i>Beauveria bassiana</i>	10 ⁵	GU566276.1	99	–
<i>Beauveria geodes</i>	10 ⁴	U19037.1	98	968
<i>Geomyces pannorum</i> var. <i>asperulatus</i>	10 ³	AJ938166.1	99	–
<i>Hypocrea pachybasioides</i>	10 ³	FJ860796.1	100	–
<i>Metarhizium anisopliae</i> var. <i>anisopliae</i>	10 ³	EU307900.1	99	950
<i>Mortierella humilis</i>	10 ⁵	AJ878778.1	99	967
<i>Mortierella macrocystis</i> (1)	10 ³	AJ878782.1	98	–
<i>Mortierella macrocystis</i> (2)	10 ³	AJ878782.1	99	965
<i>Mortierella</i> sp. (1)	10 ⁵	EU240040.1	99	–
<i>Mortierella</i> sp. (2)	10 ⁴	EU240040.1	99	–
<i>Penicillium canescens</i>	10 ⁵	DQ658168.1	100	–
<i>Penicillium spinulosum</i>	10 ⁵	GU566247.1	99	–
<i>Scytalidium lignicola</i>	10 ³	DQ093703.1	99	955
<i>Trichocladium opacum</i>	10 ⁴	FN386299.1	100	958
<i>Trichoderma viride</i>	10 ⁴	FJ481123.1	99	–
<i>Umbelopsis ramanniana</i>	10 ³	DQ888724.1	99	–
Soil1, RP				
<i>Beauveria bassiana</i>	10 ³	GU566276.1	99	960
<i>Geomyces pannorum</i>	10 ³	FJ590611.1	99	947
<i>Geomyces pannorum</i> var. <i>asperulatus</i>	10 ³	AJ938166.1	99	962
<i>Geomyces vinaceus</i> (1)	10 ⁵	AJ608972.1	100	–
<i>Geomyces vinaceus</i> (2)	10 ⁵	AJ608972.1	99	–
<i>Hypocrea pachybasioides</i> (1)	10 ³	AY240841.1	99	–
<i>Hypocrea pachybasioides</i> (2)	10 ⁴	AY240841.1	99	944
<i>Hypocrea</i> sp.	10 ³	EU294196.1	99	–
<i>Hypocrea viridescens</i>	10 ³	EU871022.1	99	–
<i>Mortierella alpina</i>	10 ⁴	FJ161918.1	100	959
<i>Mortierella gamsii</i>	10 ⁴	DQ093723.1	100	948
<i>Mortierella macrocystis</i> (1)	10 ⁵	AJ878782.1	87	–
<i>Mortierella macrocystis</i> (2)	10 ⁵	AJ878782.1	99	–
<i>Mortierella</i> sp. (1)	10 ⁵	FJ810149.1	99	–
<i>Mortierella</i> sp. (2)	10 ³	FJ810149.1	99	–
<i>Mortierella</i> sp. (3)	10 ³	FJ810149.1	100	–
<i>Mortierella</i> sp. (4)	10 ³	GQ302682.1	99	–
<i>Paecilomyces carneus</i>	10 ⁵	AB258369.1	99	961
<i>Penicillium</i> sp.	10 ⁵	FJ379821.1	99	–
<i>Penicillium spinulosum</i>	10 ⁴	GU566247.1	99	–
<i>Trichosporon porosum</i>	10 ⁵	AF414694.1	99	949
<i>Umbelopsis ramanniana</i> (1)	10 ⁵	DQ888724.1	99	–
<i>Umbelopsis ramanniana</i> (2)	10 ⁵	EU484205.1	97	941
<i>Umbelopsis ramanniana</i> (3)	10 ⁴	DQ888724.1	98	–
Zygomycete	10 ⁵	EF152531.1	90	–

Continued overleaf

Table 3. Continued

Isolate ^a	CFU g ⁻¹ dry soil	Homolog sequence in NCBI	Max identity, %	Strain in MSCL ^b
Soil2, AVP				
<i>Beauveria geodes</i>	10 ³	U19037.1	99	–
<i>Lecythophora mutabilis</i>	10 ⁴	HM036599.1	99	–
<i>Mortierella</i> sp.	10 ⁴	FJ810149.1	99	–
<i>Penicillium montanense</i> (1)	10 ⁵	AF527058.1	100	–
<i>Penicillium montanense</i> (2)	10 ⁵	AF527058.1	100	–
<i>Penicillium montanense</i> (3)	10 ⁴	AF527058.1	100	–
<i>Penicillium montanense</i> (4)	10 ³	AF527058.1	100	942
<i>Penicillium spinulosum</i>	10 ⁴	GU566247.1	99	–
Pezizomycete	10 ⁵	GQ153212.1	82	–
<i>Pochonia bulbillosa</i> (1)	10 ³	AB378551.1	99	–
<i>Pochonia bulbillosa</i> (2)	10 ⁴	AB378551.1	99	964
<i>Pseudeurotium bakeri</i>	10 ⁵	HM036612.1	100	–
<i>Tolypocladium inflatum</i>	10 ⁵	GU354362.1	99	970
<i>Trichoderma viride</i> (1)	10 ³	FJ872073.1	99	946
<i>Trichoderma viride</i> (2)	10 ⁴	FJ481123.1	99	–
<i>Umbelopsis isabellina</i>	10 ³	AJ876493.1	97	951
Soil2, RP				
<i>Aspergillus cervinus</i>	10 ³	AY373845.1	98	963
<i>Beauveria caledonica</i>	10 ³	AY532003.1	99	971
<i>Geomyces pannorum</i> var. <i>asperulatus</i>	10 ³	AJ938166.1	99	–
<i>Hypocrea pachybasioides</i> (1)	10 ⁴	AY240841.1	99	–
<i>Hypocrea pachybasioides</i> (2)	10 ³	GU062213.1	98	–
<i>Hypocrea pachybasioides</i> (3)	10 ³	AY240844.1	99	–
<i>Lecythophora mutabilis</i>	10 ³	HM036599.1	99	956
<i>Mortierella</i> sp. (1)	10 ⁴	EU240040.1	91	–
<i>Mortierella</i> sp. (2)	10 ³	FJ810149.1	100	–
<i>Mortierella</i> sp. (3)	10 ⁵	EU240039.1	99	–
<i>Penicillium spinulosum</i>	10 ⁵	GU566247.1	99	952
<i>Podospora appendiculata</i>	10 ³	AY999126.1	92	953
<i>Pseudeurotium bakeri</i> (1)	10 ³	HM036612.1	99	–
<i>Pseudeurotium bakeri</i> (2)	10 ³	HM036612.1	100	943
<i>Rhizosphaera kalkhoffii</i>	10 ⁵	AY183366.1	100	954
<i>Tolypocladium inflatum</i>	10 ⁴	GU354362.1	99	957
<i>Trichoderma asperellum</i> (1)	10 ³	FJ605246.1	99	966
<i>Trichoderma asperellum</i> (2)	10 ³	FJ605246.1	99	–
<i>Trichoderma viride</i> (1)	10 ³	FJ481123.1	99	969
<i>Trichoderma viride</i> (2)	10 ³	FJ481123.1	99	–
<i>Trichoderma viride</i> (3)	10 ³	FJ481123.1	99	–
<i>Trichoderma viride</i> (4)	10 ³	FJ481123.1	99	945
<i>Trichoderma viride</i> (5)	10 ³	FJ481123.1	99	–
Zygomycete (1)	10 ⁵	AM292200.1	96	–
Zygomycete (2)	10 ³	AM292200.1	96	–
Zygomycete (3)	10 ³	AM292200.1	96	–
Zygomycete (4)	10 ³	EF152547.1	92	–

^a Number in the parentheses indicates different isolates.

^b Microbial Strain Collection of Latvia.

Several sequenced *Trichoderma* isolates were obtained as sterile mycelia on agar plates and did not sporulate until the end of the laboratory experiments in May 2010. Seasonality studies of *Trichoderma* species in spruce forest soil in Canada showed that members of this genus are more influenced by the competition among soil fungi than environmental factors such as soil moisture content (Widden & Abitbol, 1980). The small proportion of *Trichoderma* spp. in the soil fungal community can explain why trees in both stands were heavily infected with *H. parviporum*.

Representatives of the genus *Mucor* were present in a low percentage: on average 1–2% in Soil1, with the exception of the upper soil layer in May (9.69%), November (21.23%), and April (11.38%) when a larger proportion of this group of moulds was found among the isolates. In Soil2 *Mucor* spp. were more abundant in May–November (on average 9.28%) in comparison with December–April (1.28%). *Gilmaniella* spp., *Geomyces* spp., and *Aspergillus cervinus* were isolated only in the RP. *Botrytis* was isolated only in the AVP. Several insect pathogens were isolated from the analysed soils: *Beauveria bassiana* (RP, AVP), *B. caledonica* (RP), *B. geodes* (AVP), and *Metarhizium anisopliae* (AVP).

Table 3 gives an overview of 84 sequenced fungal isolates (45 sporulating, 39 sterile mycelia). The sequence database of the National Center for Biotechnology Information (NCBI) and Blast algorithm were used for sequence comparison; in most cases the sequence similarity exceeded 98%. One member of every species identified was deposited in the Microbial Strain Collection of Latvia.

Sequencing revealed different composition of the communities in Soil1 and Soil2: only five species were common in both experimental plots (*B. geodes*, *G. pannorum*, *H. pachybasioides*, *P. spinulosum*, and *T. viride*). From Soil1 19 unique species were isolated and identified, but in the case of Soil2 their number was 16. The corresponding Sørensen's community similarity index C_s was 0.29. In Soil1 six species were common in the AVP and RP (*B. bassiana*, *G. pannorum*, *H. pachybasioides*, *M. macrocystis*, *P. spinulosum*, and *U. ramanniana*). The corresponding C_s was 0.48. In Soil2 five species were common in both periods (*L. mutabilis*, *P. spinulosum*, *P. bakeri*, *T. inflatum*, *T. viride*, and *U. isabellina*). The corresponding C_s was 0.48.

Results of the screening for psychrotrophic and mesophilic fungi

Several fungal species isolated from the soil samples of the RP have been detected in arctic environments or recorded as growing at low temperatures (such as 0°C): *G. vinaceus*, *G. pannorum*, *M. alpina* (Bergero et al., 1999), and also *T. porosum* (Danielson & Davey, 1973). Although all our isolates of these fungi were able to grow at 4°C, their growth was not impeded at 20°C either.

Several species were typically isolated only in the AVP; for example, *P. bulbillosa*, *S. lignicola*, *M. anisopliae* var. *anisopliae*, and *P. montanense*. From these species *M. anisopliae* var. *anisopliae* was not able to grow at low temperatures.

In laboratory conditions (in vitro) all the tested isolates showed mesophilic growth, none of them was psychrotrophic. The members of *Trichoderma/Hypocrea* had the highest growth rates among all strains at 20°C and at 4°C: 5.94–13.17 mm/day and 1.43–1.98 mm/day, respectively. Also the members of the genus *Mortierella* had high growth rates: 5.68 and 2.17 mm/day; an exception was the isolate of *Mortierella alpina* from the RP with slower growth rates: 2.24 and 0.85 mm/day. One isolate of *T. inflatum* from the RP and one isolate of the same species from the AVP showed identical growth rates at 20°C and 4°C: 1.41 ± 0.21 and 0.30 ± 0.09 mm/day, respectively. From all 29 isolates tested only *M. anisopliae* var. *anisopliae* was not able to grow at 4°C. The isolate of *R. kalkhoffii* from the RP showed identical growth rates (0.57 ± 0.16 and 0.52 ± 0.09 mm/day) at both temperatures up to the 10th day of incubation, but later it started to grow significantly faster at 20°C (1.08 ± 0.12 and 0.47 ± 0.06 mm/day). The comparative growth rate of the isolated strains in laboratory experiments is indicative. It may not reflect the actual growth rate of the same strains in soil (in situ), although the correlation between in vitro and in situ data is expected to be positive; for example, the intensive growth rate of *Trichoderma*, which was found in our experiments, is the basis for the use of these fungi in plant protection in situ.

Total soil DNA amount and results of qPCR

The success of the molecular methods depends on the quantity and quality of the extracted total soil DNA. Our results of the total DNA amount of $10.03 \pm 4.29 \mu\text{g g}^{-1}$ dry soil in Soil1 and $18.17 \pm 9.59 \mu\text{g g}^{-1}$ dry soil in Soil2 are similar or higher than those found in other investigations where MoBio kits have been used: $11.1 \pm 0.3 \mu\text{g g}^{-1}$ dry agricultural soil (Thakuria et al., 2008), $1.46 \pm 3.7 \mu\text{g g}^{-1}$ dry podzolic soil (Carrigg et al., 2007); $1.06 \pm 0.35 \mu\text{g g}^{-1}$ dry wetlands soil (Ning et al., 2009). Further molecular analysis of the DNA isolated from soil may be impeded by the presence of various inhibitors; many of them belong to humic compounds (Ning et al., 2009). We used Bovine Serum Albumin as a scavenger of potential inhibitors in the PCR reactions.

Changes in the amount of the total soil DNA in soil samples at the experimental plots are shown in Fig. 5a, b. The total soil DNA amount was similar at both sampling depths and in the RP and AVP, but it differed significantly ($F = 5.009$; $p < 0.01$) between the experimental plots: $10.03 \pm 4.29 \mu\text{g g}^{-1}$ dry soil in Soil1 and $18.17 \pm 9.59 \mu\text{g g}^{-1}$ dry soil in Soil2.

Changes in the amount of the fungal DNA during the analysed period are shown in Fig. 5c, d. No significant differences were observed at either analysed soil depth or in either experimental plot. However, in both experimental plots there were statistically significant differences between the AVP and RP. In Soil1 the fungal DNA amount was significantly ($F = 8.854$; $p = 0.013$) lower ($8.08 \pm 5.97 \text{ ng g}^{-1}$ dry soil, excluding the value of the depth 10–30 cm in September) during the AVP than during the RP ($112.40 \pm 61.55 \text{ ng g}^{-1}$ dry soil, excluding the

Seasonal changes in forest soil microbial populations

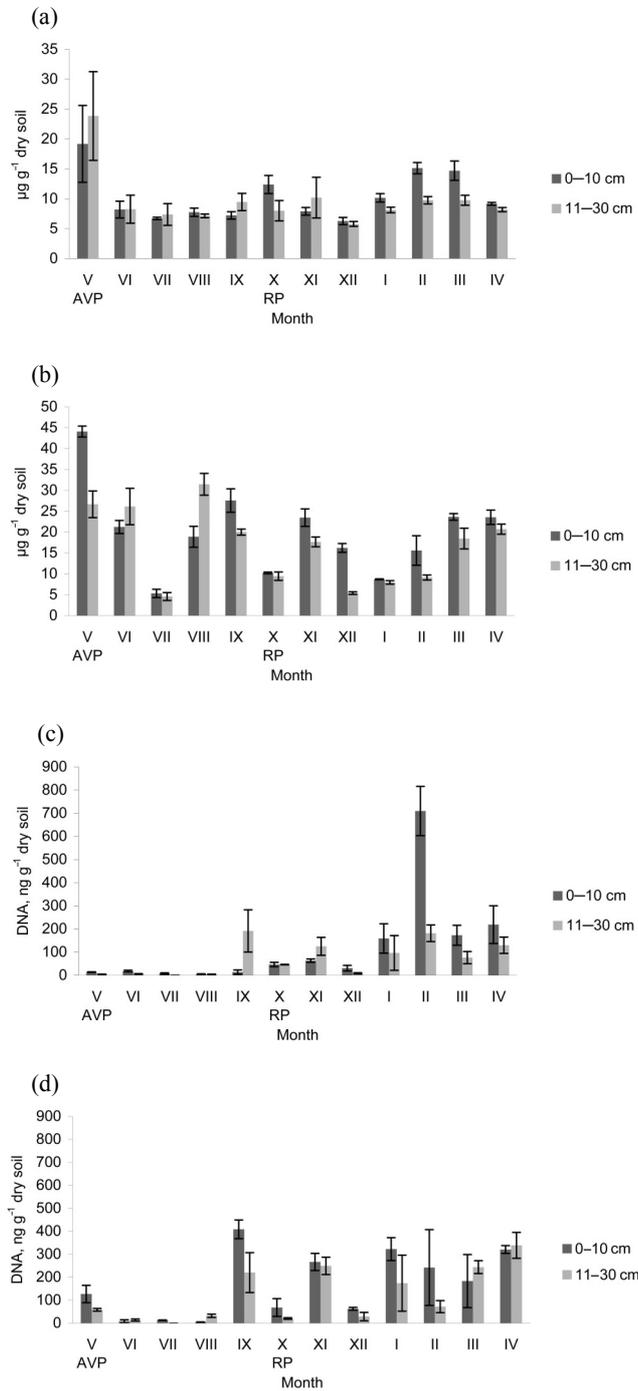


Fig. 5. Amount of total soil DNA (a, b) extracted with PowerSoil™ DNA Isolation Kit and total fungal DNA (c, d) estimated using qPCR in sampling plots Soil1 (a, c) and Soil2 (b, d) at two depths ($\pm\text{SD}$; $n = 3$) in the investigated period.

values of the upper soil layer in February and the lower soil layer in December). Similarly, the fungal DNA amount in Soil2 was significantly higher during the RP than during the AVP ($F = 1.331$; $p = 0.035$). During the RP the fungal DNA amount constituted on average 1.37% of the total soil DNA in Soil1 and 1.16% in Soil2. In the AVP the fungal DNA amount constituted a much lower proportion of the total soil DNA: on average 0.26% in Soil1 and 0.39% in Soil2. In both experimental plots the differences between the AVP and RP were statistically significant ($F = 3.125$; $p = 0.003$ and $F = 3.382$; $p = 0.003$).

The total amount of the fungal DNA increased at lower air temperatures in both experimental plots. This is in line with other investigations where it was determined that fungal populations dominate under the snow cover and that winter fungal populations are adapted to colder temperatures and more complex substrates (Lipson et al., 2002). It seems that cultivation independent methods such as fungal abundance in terms of hyphal length or molecular methods are more suitable for the studies of seasonal changes of fungal populations than the plate count method.

Our results agree with the observations that microbial biomass and activity increase to maximal levels in late winter under the snow and then decline during snowmelt due to the limitation of carbon sources (Brooks et al., 1998; Lipson et al., 1999, 2000; Schmidt et al., 2004). Our data demonstrated that during January and February when the soil was covered with snow the fungal DNA concentration values were higher than in the samples from March when snow was melting away. Also other analysed parameters showed a similar tendency. The best examples are the number of CFF in Soil1 in March (Fig. 3a), the number of CFU of yeasts and maltose utilizing bacteria in Soil1 (Fig. 3c), the total soil DNA amount in Soil1 (Fig. 5a), fungal DNA amount in Soil1 (Fig. 5d), and H' in both experimental plots (Fig. 6b) in March. This tendency is slightly different in Soil2, which remained unfrozen during the winter. In this experimental plot the values of the analysed indicators continued to increase also during March and decreased only in April.

The DNA amount of *Trichoderma* spp. was similar at both sampling depths in the experimental plots, and did not differ significantly between the AVP and RP (Fig. 6). However, it was significantly ($F = 32.088$; $p = 0.015$) higher in Soil2 (3.64 ± 3.10 ng g⁻¹ dry soil) than in Soil1 (0.72 ± 0.44 ng g⁻¹ dry soil, except the values in March). Both experimental plots had on average a higher percentage of *Trichoderma* spp DNA of the total fungal DNA in the AVP compared to the RP, but these differences were statistically not significant (6.68% and 4.50% versus 1.81% and 1.43% in Soil1 and Soil2, respectively). *Trichoderma* spp. DNA was found also in soil samples in which no CFU of *Trichoderma* genus were detected.

In general the correlation between the results of the conventional plate count (number of CFF) and the amount of fungal DNA was weak (correlation coefficient 0.18). This observation may reflect the accumulation of unculturable or psychrophilic fungi that enjoy optimum growth conditions under the snow

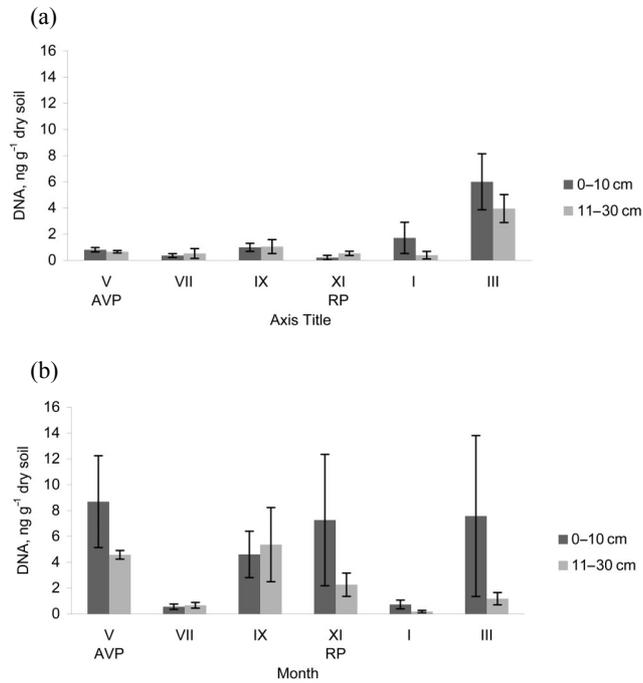


Fig. 6. Amount of *Trichoderma* spp. DNA estimated using qPCR in sampling plot Soil1 (a) and Soil2 (b) at both sampling depths (\pm SD; $n = 3$) in every second month.

cover in the presence of decaying plant litter. Their DNA could be amplified and identified, but we still could not grow the corresponding fungi in standard laboratory conditions.

Results of ARDRA

Typical results of ARDRA in 6% polyacrylamide gel electrophoresis are shown in Fig. 7 and changes of the Shannon–Weaver diversity indices of fungi in the experimental plots are given in Fig. 8. Fungal diversity indices were similar in both experimental plots and at both sampling depths. There were no statistically significant differences between the AVP and RP, but in some months (June, September, January) the fungal diversity was significantly lower than on average. The weak potential of molecular analysis derived diversity indices to evaluate the response of microbial populations to environmental factors has been recognized previously (Hartmann & Widmen, 2006). However, comparison of differences in the fungal community structure using cluster analysis (Fig. 9) showed tendencies for the soil samples from the RP to form a separate cluster in the case of Soil1 and several clusters in the case of Soil2. This means that the fungal communities were structurally different in the RP and AVP.

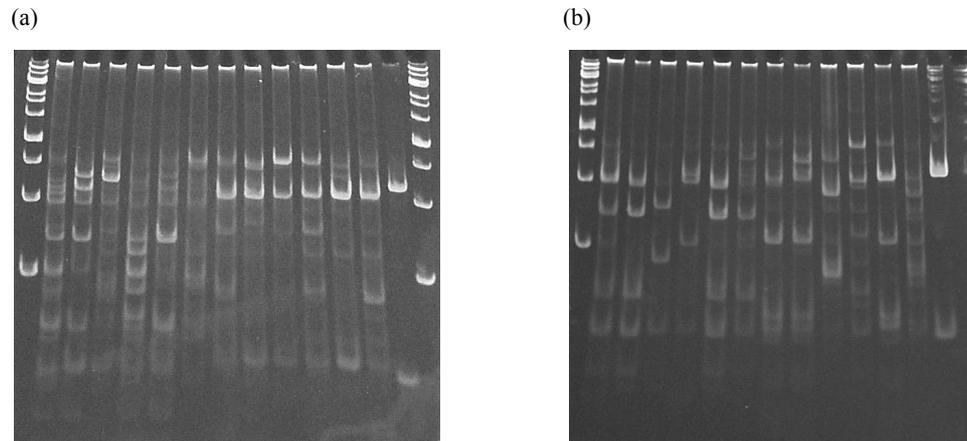


Fig. 7. Results of ARDRA in 6% polyacrylamide gel electrophoresis (August (a) and February (b)). Lanes 1–6 Soil1, 7–12 Soil2. +K, restriction products of *Heterobasidion parviporum* MSCL 981 DNA amplification. M, Gene Ruler 1 kb DNA Ladder (Fermentas).

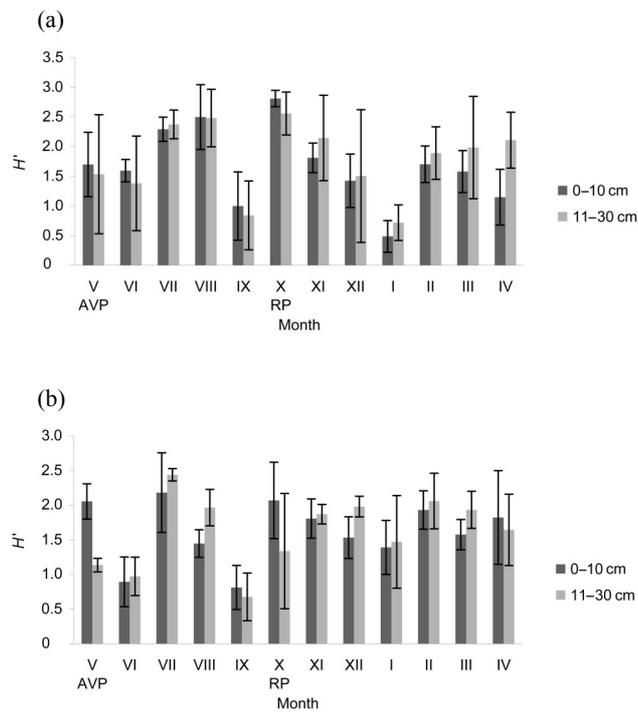


Fig. 8. Changes of the Shannon–Weaver diversity indices H' of fungi in sampling plots Soil1 (a) and Soil2 (b) at two depths (\pm SD; $n = 3$) in the investigated period. Indices were obtained using ARDRA in 6% polyacrylamide gels.

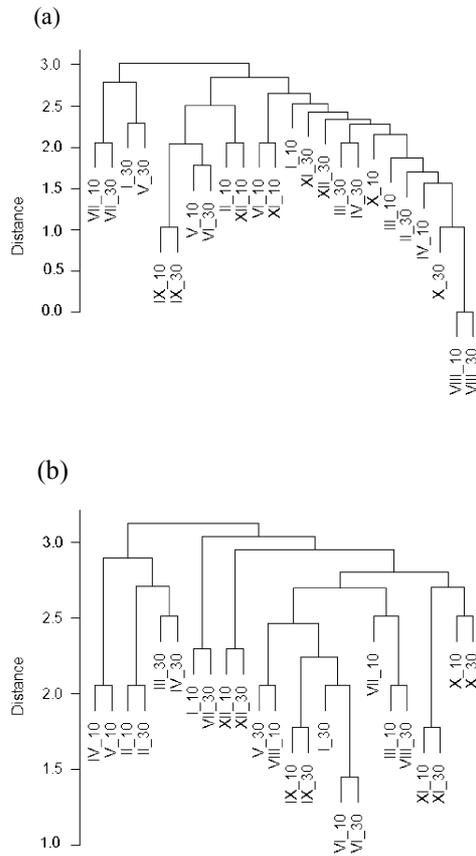


Fig. 9. Cluster analysis of seasonal fungal community structure differences in Soil1 (a) and Soil2 (b).

Results of the statistical analysis

In temperate regions the highest input of litter to the soil occurs in autumn when many plants are senescing and plant roots are dying. The winter microbial population starts to decompose the litter and to use phenolic carbon sources as well as to immobilize nitrogen. During snowmelt these populations die off and the accumulated nitrogen is used for plants and summer microbial populations. In cold systems the limiting factor for microbial growth is the availability of water but not the temperature. Under the snow even when the air temperature is significantly below 0°C unfrozen water remains around the soil particles (Schmidt & Lipson, 2004).

Results of multiple regression analyses are listed in Table 4. According to these analyses, the number of CFF was significantly affected by soil moisture

Table 4. Results of multiple regression analyses

Factor	CFF	Yeasts and maltose utilizing bacteria	Total number of micro-organisms	Total amount of soil DNA	H'	Total amount of fungal DNA	Total amount of <i>Trichoderma</i> spp. DNA
Soil pH	NS	0.020 ^b	0.005 ^b	NS	NS	NS	NS
Average air temperature	0.01	NS	NS	0.020	NS	<0.001 ^b	NS
Soil moisture content	<0.001	NS	NS	<0.001	NS	<0.001	<0.001
Multiple R-squared	0.387	0.459	0.489	0.551	–	0.537	0.627
p-Value of the model	<0.001	<0.001	<0.001	<0.001	–	<0.001	<0.001

^b – negative impact; NS, not significant; –, not applicable.

content (Pearson correlation coefficient $r = 0.59$; $p < 0.001$) and to some extent also by average air temperature ($r = 0.23$; $p = 0.01$). When both experimental plots were analysed separately, the data showed no correlation between air temperature and the number of CFF in Soil1, but in Soil2 there was a weak correlation ($r = 0.48$; $p < 0.001$). The significant positive impact of soil moisture content on the number of CFF explains the low number of fungal isolates from Soil2 in July, October, and January: during these months the soil in this experimental plot was very dry, the structure of the soil (Table 2) facilitated drainage and did not allow accumulation of the water precipitated during previous months.

According to the results of multiple regression analyses, the impact of soil moisture content and average air temperature on the number of yeasts and maltose utilizing bacteria and also on the total number of culturable microorganisms was significant, and high soil moisture together with high average air temperature can explain the increased numbers of CFU obtained in August. When the soil moisture content was higher, we observed higher numbers of CFF as well as a higher total amount of the fungal DNA, including *Trichoderma* spp. DNA. The numbers of yeasts and maltose utilizing bacteria were not so affected by soil moisture as the CFF, with the exception of the combination of high soil moisture with high air temperature as in the case of Soil2 in August.

The soil pH varied in the analysed soil horizons from 2.42 to 3.68. In Soil2, which had a lower pH, the amount of yeasts and maltose utilizing bacteria was on average higher: 3.13×10^6 CFU g⁻¹ of dry soil in the upper soil layer and 9.83×10^5 CFU g⁻¹ of dry soil in the deeper soil layer versus 1.26×10^6 CFU g⁻¹ of dry soil and 6.96×10^5 CFU g⁻¹ of dry soil in Soil1. The soil pH, which was different between the experimental plots, significantly influenced the number of yeasts and maltose utilizing bacteria and the total number of soil microorganisms (data not shown). The total amount of soil DNA was influenced by the soil moisture ($r = 0.65$; $p < 0.001$) and average air temperature ($r = 0.22$; $p < 0.001$).

When data of the experimental plots were analysed separately, no correlation was found between the air temperature and soil moisture and the total amount of soil DNA in Soil1, while in Soil2 there was a weak correlation of the total amount of soil DNA with air temperature ($r = 0.42$; $p < 0.001$) and a moderately strong correlation with soil moisture ($r = 0.78$; $p < 0.001$). According to the results of *t*-test, these differences were not statistically significant, probably due to the fact that in Soil2 the CFU counts were significantly different between the AVP and RP.

Total amount of fungal DNA was significantly negatively affected by air temperature ($r = -0.60$; $p < 0.05$) (Fig. 10a) and positively affected by soil moisture content ($r = 0.44$; $p < 0.001$). Higher concentrations of fungal DNA at lower air temperatures were found in both experimental plots. The total amount of *Trichoderma* spp. DNA was affected only by soil moisture content ($r = 0.76$; $p < 0.001$) (Fig. 10b).

Fungal diversity expressed as the Shannon–Weaver diversity index H' was not influenced by any of the analysed factors.

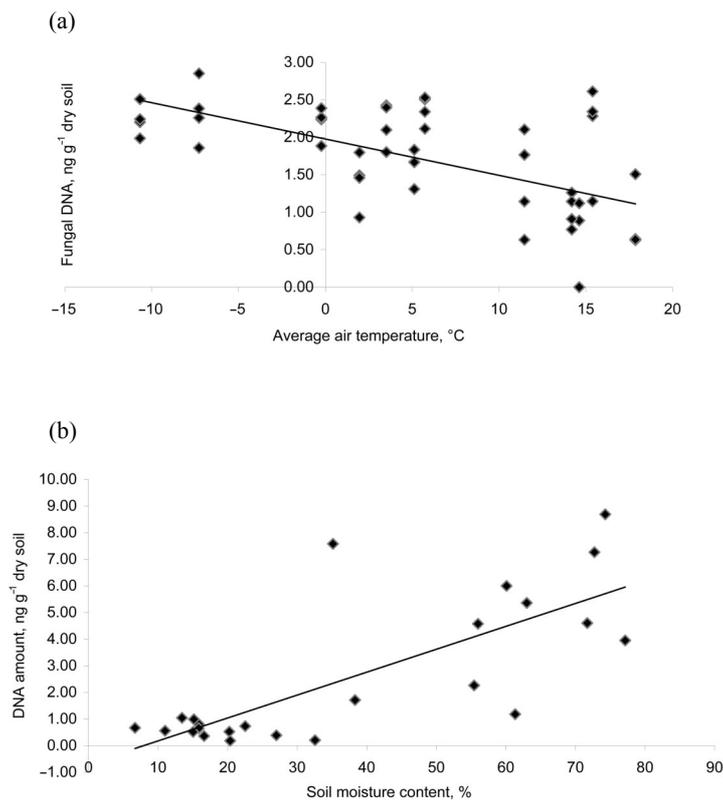


Fig. 10. Charts of correlations between average air temperature and total fungal DNA amount ($R^2 = 0.36$) (a) and between soil moisture content and *Trichoderma* spp. DNA amount ($R^2 = 0.58$) (b).

CONCLUSIONS

We can conclude that the microbial communities of both closely located experimental plots showed a different response to changing environmental factors during the AVP and RP. This can be explained by the fact that the air temperature and precipitation differed between the plots due to the differences in their micro-habitat topography: soil type, texture, composition of chemical elements, which influenced the changes of soil moisture content and properties of soil horizons.

In the sod-podzolic soil (Soil1) only the fungal DNA amount varied significantly during the analysed period: it was significantly higher in the RP in comparison to the AVP.

In the illuvial humus podzol (Soil2) the number of culturable filamentous fungi, yeasts, and maltose utilizing bacteria as well as the abundance of *Penicillium* spp. were significantly higher in the AVP but the fungal DNA amount was significantly higher in the RP.

Several fungal species (*Geomyces vinaceus*, *G. pannorum*, *Mortierella alpina*, *Trichosporon porosum*) isolated from the soil samples of the RP have been detected in arctic environments or recorded as growing at low temperatures such as 0°C. Although all our isolates of these fungi were able to grow at 4°C, they did not show any psychrotrophic growth characters.

Several species were typically isolated only in the AVP (*Pochonia bulbillosa*, *Scytalidium lignicola*, *Metarhizium anisopliae* var. *anisopliae*, and *Penicillium montanense*). From these species *M. anisopliae* var. *anisopliae* was not able to grow at 4°C. In general the growth rate was genus and species dependent.

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Sesoonsed muutused parasvöötme kuusemetsa mulla mikroobikoosluses

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Käesoleva uurimistöö eesmärgiks oli uurida sesoonsed muutusi parasvöötme kuusemetsa mulla mikroobikoosluses, kasutades traditsioonilisi ja molekulaarseid mikrobioloogilisi meetodeid. Materjal mikrobioloogilisteks analüüsideks koguti mulla kahest ülemisest horisondist kahelt eri tüüpi mullaga hariliku kuuse alalt 12 kuu pikkuse perioodi jooksul. Mulla mikroobikooslus oli seotud erinevate keskkonnateguritega uuritud aladel. Vegetatsiooniperioodiga (maist septembrini) võrreldes oli leetmulla korral seente DNA kogus mullas oluliselt suurem puhkeperioodil (oktoobrist aprillini) ja *Penicillium*'i perekonna liikide arv oli suurem vegetatsiooniperioodil. Huumusilluviaalse leedemulla puhul oli pärmide, *Penicillium*'i perekonna liikide ja kultiveeritavate niitjate seente arvukus suurem vegetatsiooniperioodil. 84 seeneisolaadi ITS-i järjestuste analüüs näitas kahe uuritud ala seenekoosluse suurt erinevust (Sørenseni indeks 0,29), kuid sesoonne erinevus oli väiksem (Sørenseni indeks 0,48). Puhkeperioodil kogutud seeneisolaadid olid võimelised kasvama 4°C juures, kuid ükski neist isolaatidest ei olnud psührotroofne tüvi.