Chemical and Microbial Properties of Sandy Mine Soils Afforested with Scots Pine and Silver Birch

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Abstract

The objective of this study was to compare chemical and microbial properties of sandy mine soils under young Scots pine, silver birch, and mixed pine-birch forest stands. The measured properties included the contents of organic C (C_{org}) and total N (N_t), the C_{org}-to-N_t ratio, pH, microbial biomass, basal respiration, and activities of dehydrogenase, acid phosphomonoesterase, and urease. Community level physiological profiles (CLPPs) of soil bacteria were determined with Biolog® test and genetic profiles with the DGGE method. Scots pine and silver birch did not affect the C_{org} and N_t contents in the studied mine soils. The soil under birch contained larger and more active microbial biomass than the soil under pine. Under the mixed stand, most of the microbial properties were intermediate between the pine and the birch stand. The DGGE profiling indicated different composition of soil bacteria under the birch stand compared to the other stands. Differences in CLPPs were less pronounced, probably due to functional redundancy of soil bacteria.

Keywords: mine soils, microbial biomass, enzyme activities, CLPP, DGGE

Introduction

Areas destroyed by surface mining need to be reclaimed and afforestation is a common way of reclamation of post-mining barrens. The most important goals of reclamation for forestry include restoration of soil cover and reestablishment of properly functioning nutrient cycling. The post-mining barrens are often built of materials that do not contain organic matter and exhibit extremely low microbial activity [1]. Therefore, measurements of organic matter content and soil microbial properties have been proposed for estimation of reclamation success. There are a number of soil chemical and microbial properties that are used for this purpose. The most common properties include contents of organic C, total N, pH, microbial biomass, basal respiration and activities of different soil enzymes [2, 3]. In recent years various measures of soil microbial community structure have been proposed as useful indicators of reclamation success [4, 5], as they may provide information relevant to the functioning of soils [6].

Areas destroyed by mining are often hostile environments for the plated trees due to unfavorable physical and chemical properties of soil substrates. Therefore, only some tree species can be used for afforestation of post-mining barrens. Scots pine (*Pinus sylvestris*) and silver birch (*Betula pendula*) are often used to afforest areas destroyed by mining because they are able to survive on extremely poor sites. These two tree species are known to produce litter of different chemical composition and differently affect microbial properties in natural soils [7-9]. However, mine soils represent a specific environment [10] and therefore the effect of tree species on the microbial properties of the mine soils may differ from the effect known for the natural forest soils.

The objective of this study was to compare some chemical and microbial properties of sandy mine soils under young Scots pine, silver birch, and mixed pine-birch forest stands.

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Material and Methods

Study Site

Our study was carried out in Upper Silesia, Poland (19º26'E; 50º16'N) within the area of the Szczakowa open-cast sand quarry. The climate in the study area is temperate, with mean annual precipitation of *ca* 700 mm and mean annual temperature of 8ºC. The sand deposits extracted in the quarry are fluvioglacial Quaternary sediments of pre-Quaternary morphological depression.

The Szczakowa open-cast quarry has been extracting sand since 1954. Mining created an open cast 5-25 m deep, covering over 2,700 ha. Since the late 1950s it has been reclaimed and afforested. The reclamation procedure included forming and leveling off the surface and adding an organic amendment (approx. 300 $m^3 \cdot ha^{-1}$). The added amendment was a mixture of forest floor (O horizon) and mineral horizons (horizons A_h, E, and partly B), with average organic C content of 0.3-1.0%, collected from forest soils in areas to be mined. Then lupine (*Lupinus luteus* L.) was cultivated for two years. The lupine cultivations were fertilized with NPK (140 kg N ha^{-1}, 300 kg P_2O_5 ha^{-1}, and 180 kg K_2O ha^{-1}). After two years, the lupine biomass was ploughed into the soil as green manure and the sites were afforested with 1-year-old seedlings of Scots pine (*Pinus sylvestris*) and silver birch (*Betula pendula*).

Soil Sampling

Samples of the mineral soil (0-5 cm) were taken in November 2009 from the sites covered by pure Scots pine (*Pinus sylvestris*) and silver birch (*Betula pendula*) stands and from mixed pine-birch stand (share of each tree species – 50%). The sampled forest stands were 23-24 years old. The number of stems per ha was *ca* 2000. At each site six mixed samples were taken. Each mixed sample consisted of five subsamples (area of each subsample = 0.16 m^2) located at the corners and in the middle of a 3 x 3 m square. The samples were sieved (2 mm mesh) and divided into two parts. One part was air-dried and used for physical, physico-chemical, and chemical analyses, and the other one was stored field-moist at 4ºC and used for microbial and biochemical analyses. Prior to microbial analyses the samples were adjusted to 50% of maximum water holding capacity (WHC) and pre-incubated at 22ºC for six days.

Physical, Physico-Chemical, and Chemical Analyses

The pH of the samples was measured in 1 M KCl solution (soil:liquid ratio 1:2.5, w:v) with a digital pH-meter (CP-401, ELMETRON). Content of organic C (C_{org}) and total N (N_t) was determined by dry combustion with a CN analyzer (Vario Max, Elementar Analysensysteme GmbH). The soil texture of the mineral soils was determined hydrometrically according to the Casagrande-Prószyński method.

Determination of Microbial Biomass and Basal Respiration

To measure basal respiration (RESP) and microbial biomass (C_{mic}), samples (50 g d.w.) unamended for RESP measurements and amended with 200 mg glucose monohydrate for C_{mic} measurements were incubated at 22°C in gas-tight jars. The incubation time was 24 hours for determination of RESP and 4 hours for C_{mic}. The jars contained small beakers with 5 ml 0.2 M NaOH to trap the evolved CO_2. After the jars were opened, 2 ml 0.5 M $BaCl_2$ was added to the NaOH; the excess of hydroxide was titrated with 0.1 M HCl in the presence of phenolphthalein as indicator. C_{mic} was calculated from the substrate-induced respiration rate according to the equation given by Anderson and Domsch [11]:

$$C_{mic} \ [mg \cdot g^{-1}] = 40.04 \ y + 0.37$$

...where y is ml CO_2 $h^{-1} \cdot g^{-1}$.

Soil Enzyme Activities

Dehydrogenase activity was determined according to von Mersi [12]. The soil samples (1 g d.w.) were mixed with 1.5 ml Tris buffer (pH 7) and 2 ml 0.5% INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride) solution, and incubated at 40ºC for 2 h. The reduced iodonitrotetrazolium formazan (INTF) was extracted with 10 ml dimethyloformamid/ethanol (1:1) and measured photometrically at 464 nm. Dehydrogenase activity was expressed as µg INTF $g^{-1} \cdot h^{-1}$.

Acid phosphomonoesterase activity was measured as described by Margesin [13]. The soil samples (1 g d.w.) were mixed with 1 ml disodium p-nitrophenyl phosphate solution (115 mM) and 4 ml buffer solution (pH 6.5) and incubated at 37ºC for 1 hour. The p-nitrophenol released by phosphatase activity was extracted and colored with NaOH and determined photometrically at 400 nm. Acid phospho-monoesterase activity was expressed as µg p-NP $g^{-1} \cdot h^{-1}$.

Urease activity was determined as described by Kandeler [14]. The soil samples (5 g d.w.) were mixed with 2.5 ml urea (720 mM) and 20 ml borate buffer (pH 10) and incubated at 37ºC for 4 hours. The released ammonium was extracted with acidified potassium chloride solution, coloured in the modified Berthelot reaction and measured photometrically at 690 nm. Urease activity was expressed as µg N $g^{-1} \cdot h^{-1}$.

Community Level Physiological Profiles (CLPP)

The physiological profiles of the bacterial communities were analyzed using Biolog® Ecoplates [15]. Samples (10 g d.w.) were shaken for 60 min. in 20 ml of a 10 mM Bis-Tris solution (pH 7) and allowed to settle for 30 min. Then the extracts containing microbes were decanted, and subsamples of the extracts (2 ml) were immediately frozen in liquid nitrogen and stored at -70ºC until analysis. In order to

ensure similar inoculum density [16], prior to analysis the thawed extracts were diluted with Bis-Tris solution to obtain 0.5 µg C_{mic} in 1 ml solution. The solutions were inoculated on microplates (100 µl per well) and incubated at 22ºC. Substrate utilization was monitored by measuring light absorbance at 590 nm. The first measurement was made immediately after inoculation, and the subsequent ones at 12 h intervals for six days. The readings for individual substrates were corrected for background absorbance by subtracting the absorbance of the control (water) well and the absorbance of the first reading. The corrected absorbance values were used to calculate the area under the absorbance curve (AUC). The calculated AUC values were standardized by dividing them by the average area under the curve (AAUC) and used in statistical analyses [16]. AAUC was used to express overall microbial activity on the plates.

Bacterial Community Structure

The bacterial community structure was studied by polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE). DNA extraction was performed using modified protocol of Yeates et al. [17]. The protocol was enriched with the step of three cycles of freezing (-70ºC for 20 min.) and thawing (100ºC for 10 min.) the sample in the initial extraction buffer. Each DNA sample was extracted from 0.5 g of soil.

PCR reaction was performed in 100 µl volume. In each PCR reaction 100 ng of the extracted DNA was used. Detailed PCR conditions are described in [18] as PCR for culture-independent DGGE. Positive controls of pure *E. coli* DNA and negative controls with water were performed in each set of reactions. The following thermal cycle was performed: 5 min. at 94ºC, then seven touch down cycles of 92ºC 30 sec., A – 30 sec., 72ºC 90 sec., where A is a touch down of 61 – 56ºC. After these initial steps, 22 cycles were performed: 92ºC in 30 sec., 55ºC in 30 sec., and 72ºC in 90 sec. Finally 68ºC over seven minutes was performed. Primers used in this study were those described by Muyzer et al. [19] for region V3 of 16S rRNA bacterial gene: 357 forward primer with GC – clamp and 518 reverse primer.

DGGE was performed using the BioRad DCode system of polyacrylamide gel electrophoresis. The electrophoresis was carried out for 15.5 h on 8% polyacrylamide gels of 25 ml volume at 60ºC and 60 V. Denaturation range was from 30 to 55% on each gel. The entire volume of precipitated and concentrated PCR product was put into the wells. As a marker for gel comparisons according to bands migration the 100 bp O'Gene Ruler (Fermentas) was used on each gel in at least three wells. The gel staining was done using Roth silver staining system. Banding pattern analysis was performed using PhotoCapt software (Vilber Lourmat). Background correction was adapted to gel quality and performed as automatic baseline subtraction (valley to valley method) for each lane separately. Bands on the lanes were identified by their positions against the standard marker, and their presence (1) or absence (0) was recorded yielding a binary data matrix that was used in statistical analyses.

Calculations and Statistical Analyses

Differences in chemical and microbial properties between the forest stands were tested by one-way ANOVA. If necessary the data were transformed to fulfill the assumption of normality. The Tukey's honestly significant differences (HSD) test for multiple comparisons was run if significant differences were found (p<0.05).

Principal component analysis (PCA) performed on covariance matrix was used to investigate Biolog® data. PCA reduces the number of independent variables to a smaller number of new variables called principal components (PCs). Interpretation of the PCs was based on significant factor loading of the individual substrate on each of the PCs. The functional diversity of microbial communities was calculated using Shannon's index:

$$H' = \Sigma_{i=1...n}\, p_i\, (\ln p_i)$$

...where n is the number of wells and p_i is the use of the *i*th substrate (AUC value) as a proportion of the sum of the use of all substrates of a plate.

The non-metric multidimensional scaling analysis (NMDS) with Bray-Curtis index as a measure of similarity was used to compare DGGE profiles of soil bacterial communities under different forest stands [20, 21]. The number of dimensions in NMDS maps was two or three, depending on the fit between similarity matrix and NMDS representation indicated by a stress value [20, 21]. The stress value lower than 0.2 indicates good representation by the NMDS maps of the information included in the rank similarity matrix.

One-way Analysis of Similarities (ANOSIM) was used to compare the genetic structures of soil bacterial communities under different tree species. The ANOSIM is nonparametric permutation procedure that compares between-groups and within-groups dissimilarities [22]. The procedure calculates R statistic that ranges from -1 to 1, with R=0 indicating completely random grouping and R=1 indicating that all replicates of a sampling site are more similar to each other than to replicates of any other site. Global R value was used to express overall dissimilarity between the stands. The significant global R values indicated the R value to differ significantly from 0, suggesting that the compared sites are significantly dissimilar. Dissimilarities between the stands were tested in pairwise comparisons and their significance was assessed according to the Hochberg-Bonferroni procedure [23].

Multivariate analyses were performed using PAST (Paleontological Statistics) software. For ANOVA Statgraphics Plus version 5.1 (Manugistics Inc.) was employed.

Results

Physical and Chemical Properties

There were some differences in soil texture under the studied forest stands (Table 1). However, all the soils were classified as sands (USDA classification).

Table 1. Mean values (n=6) and standard deviations for some physical and chemical properties of soils under the pine, birch, and mixed pine-birch forest stands.

Forest stand	Sand (%)	Silt (%)	Clay (%)	C_{org} (mg·g^{-1})	N_t (mg·g^{-1})	C_{org}-to-N_t	pH
Birch	93±3 b	5±2 a	2±1 a	5.0±0.9 a	0.21±0.03 a	24±3 b	3.9±0.2 b
Mixed	97±1 a	3±1 ab	0±0 b	4.5±0.8 a	0.18±0.03 a	25±2 b	4.1±0.1 a
Pine	98±1 a	2±1 b	1±1 ab	5.4±1.5 a	0.18±0.05 a	30±2 a	3.8±0.1 b

Values within a column sharing the same letter are not statistically different (p<0.05; Tukey's HSD test).

The contents of C_{org} and N_t did not differ between the stands and averaged 4.5-5.4 mg·g^{-1} and 0.18-0.21 mg·g^{-1}, respectively. However, C_{org}-to-N_t ratio was significantly higher under the pine stand (30) than under the other stands (24-25). Soil pH was significantly higher under the mixed stand (4.1) than under the other stands (3.8-3.9).
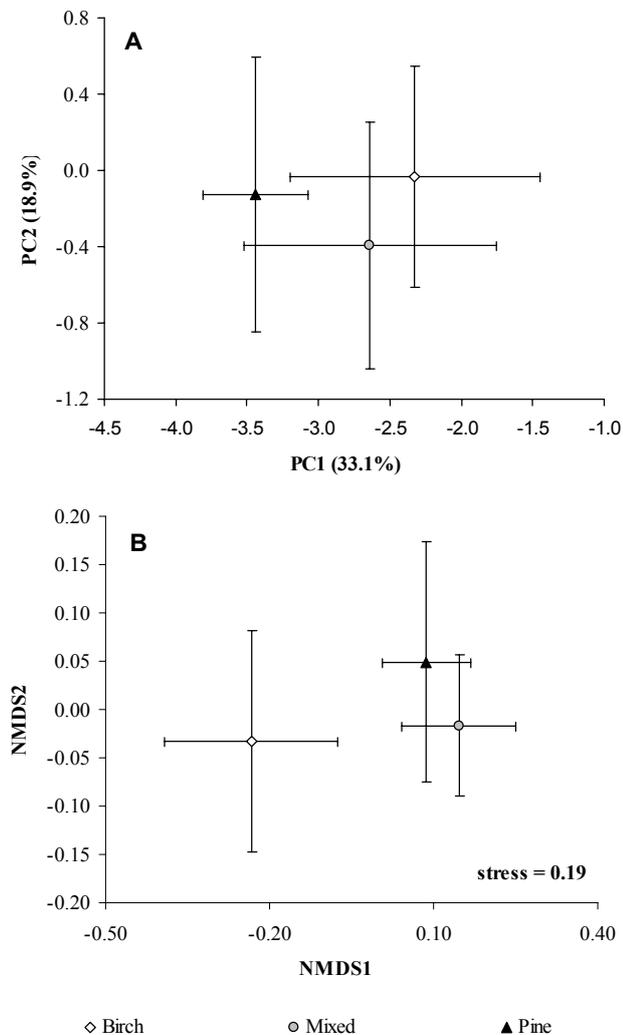

Fig. 1. Plot of principal components (A) based on Biolog® data (percent of explained variance in parentheses) and non-metric multidimensional scaling map (B) based on DGGE profiles for three analyzed forest stands. Presented are mean values (n=6); whiskers indicate standard deviations.

Gross Microbial Properties

The soil under the birch stand exhibited significantly higher RESP (9.6 µg C-CO$_2$ g^{-1} 24 h^{-1}), C_{mic}-to-C_{org} ratio (1.4%) and urease activity (4.2 µg N g^{-1}·h^{-1}) than the other stands (Table 2). The C_{mic} (68.9 µg·g^{-1}) and dehydrogenase activity (16.9 µg INTF g^{-1}·h^{-1}) were the highest under the birch stand; however, the differences were significant only compared with the pine stand. Activity of acid phosphomonoesterase was similar under all forest stands (30.4-37.4 µg p-NP g^{-1}·h^{-1}).

Physiological and Genetic Structure of Bacterial Communities

The AAUC values varied from 88 in the soil under pine to 102 in the soil under birch, but the differences were statistically not significant (Table 2). The values of Shannon's index were significantly lower under the pine stand (3.07) than under the other stands (3.16-3.19).

The first two principal components explained 51% of the variance in Biolog® data (Fig. 1). The strongest effect on PC1 was from the use of D-mannitol, β-methyl-D-glucoside, glucose-1-phosphate, and glycogen. For the PC2 the highest loadings were from phenyletylamine and β-methyl-D-glucoside. None of the PCs clearly separated the studied forest stands from each other (Fig. 1), as the PC values calculated for the particular forest stands varied over a wide range and overlapped.

The average number of bands under all stands was similar and varied from 8 to 11 (data not shown). Two NMDS dimensions were enough to obtain the stress value lower than 0.2 (Fig. 1). The NMDS and ANOSIM analysis revealed DGGE profiles under the birch stand to differ significantly from those under the pine and the mixed stand (Fig. 1, Table 3). The DGGE profiles for the pine and the mixed stands did not differ from each other.

Discussion

Experimental Design

The experimental design used in our study includes pseudoreplication, since all the sampling sites were located

Table 2. Mean values (n=6) and standard deviations of basal respiration (RESP), microbial biomass (C_{mic}), activities of dehydrogenase (DHG), acid phosphomonoesterase (AcPHP), and urease (URE), and the microbial activity on the Biolog® plates (AAUC) and Shannon diversity index (H') for the soils under the birch, pine, and mixed forest stands.

Forest stand	RESP (μg C-CO$_2$ g^{-1}·24 h^{-1})	C_{mic} (μg·g^{-1})	C_{mic}-to-C_{org} (%)	DHG (μg INTF g^{-1}·h^{-1})	AcPHP (μg p-NP g^{-1}·h^{-1})	URE (μg N g^{-1}·h^{-1})	AAUC	Shannon H'
Birch	9.6±2.7 a	68.9±30.0 a	1.4±0.6 a	16.9±2.7 a	34.3±10.4 a	4.2±1.5 a	102±16.5 a	3.19±0.09 a
Mixed	5.6±1.2 b	38.9±13.7 ab	0.8±0.2 b	15.0±3.6 ab	30.4±7.4 a	2.3±0.7 b	97±12.2 a	3.16±0.06 a
Pine	5.1±1.2 b	32.3±12.2 b	0.6±0.3 b	12.2±3.5 b	37.4±10.9 a	2.3±1.1 b	88±6.3 a	3.07±0.04 b

Values within a column sharing the same letter are not statistically different (p<0.05; Tukey's HSD test).

Table 3. ANOSIM on DGGE for the tree forest stands. The R values and level of significance (p) in parentheses. The global R value and its significance level are also presented. The R values that are significant according to the Hochberg-Bonferroni test are in bold.

	global R = 0.41 p < 0.0001	
	Mixed	Pine
Birch	**0.56** (0.008)	**0.53** (0.006)
Mixed		0.21 (0.045)

within one area [24]. Therefore, the presented data should be interpreted with caution, assuming that there are no other factors affecting the studied properties stronger than the tree species [25]. The samples were taken under the forest stands of the same age, at the sites where the same reclamation procedures were applied and all the soils belonged to the same textural group. Therefore, we assume that the major differences in the studied chemical and microbial soil properties can be attributed to the effect of tree species.

Chemical Properties

The tree species did not affect C_{org} and N_t contents in the studied soils, although silver birch and Scots pine are known to produce litter of different decomposability [9, 26, 27]. We presume that a longer time is needed for trees to cause significant difference in the contents of these elements in soils. For the 30-year-old forest stands on reclaimed post-mining barrens near Szczakowa, Chodak and Niklińska [28] reported lower C_{org} content and wider C_{org}-to-N_t ratio under the pine stand compared with the birch stand. Similar to our results, Priha and Smolander [7] reported no difference in C_{org} and N_t contents in mineral soils under 23-24-year-old birch and pine forest stands in Finland.

The C_{org}-to-N_t ratio was significantly higher under the pine stand compared with the other stands. Contrary to this result, Priha and Smolander [7] reported no difference in soil C_{org}-to-N_t ratio under the pine and birch stands. However, they studied afforested grassland soils with higher C_{org} and N_t contents than in our mine soils.

Gross Microbial Properties

The highest values of C_{mic} and RESP were determined under birch, indicating a positive effect of this tree species on soil microbial communities. Priha and Smolander [7] reported similar C_{mic} in mineral soils under 23-24-year-old pine and birch stands in Finland. Different results obtained in our study indicate that in the mine soils the effect of tree species on soil microbial biomass and respiration is stronger than in natural soils.

The C_{org} content under all forest stands was similar; therefore, the observed differences in microbial biomass and respiration resulted from different quality of soil organic matter under the studied tree species. Indeed, C_{org}-to-N_t ratio under the pine stand was wider compared with other stands. Thus, N_t deficiency might have limited the microbial growth in the soil under the pine stand.

The measured C_{mic}-to-C_{org} ratio under birch was in the range reported for natural forest soils (0.9-1.7%) [7], but it was lower under the pine and mixed stands. The larger proportion of C_{mic} in C_{org} under birch suggests better availability of soil organic matter for soil microbes compared with pine, probably resulting from different chemical composition of litter of these two tree species. Smolander and Kitunen [8] found higher amounts of dissolved organic C (DOC) in soils under birch than under pine, and Kiikkilä et al. [26] reported higher degradability of DOC from litter layer under birch compared with pine. Adamczyk et al. [27] reported significantly higher contents of tannins in soil organic horizons under silver birch compared with Scots pine, and Kanerva et al. [9] found positive correlations between condensed tannins and soil respiration and microbial biomass in the soil organic horizons in boreal forests.

The activities of dehydrogenase and urease were significantly higher under birch compared with pine. These higher activities were probably due to larger soil microbial biomass under birch, since the microbial biomass is one of the most important factors affecting soil enzyme activities [28, 29].

Physiological and Genetic Structure of Bacterial Communities

The activity on the Biolog® plates did not differ between the studied forest stands. Similar activity on the plates

under all the stands was expected, since the extracts used for the inoculation contained the same amount of microbial C. However, physiological diversity of soil microbial communities was slightly lower under the pine stand. This result corroborates with the results of our previous study [30], where relatively low physiological diversity in the reclaimed mine soil under a 20-year-old pine forest has been described.

Principal component analysis did not indicate significant differences in physiological abilities of soil bacteria under the studied stands. On the contrary, DGGE analysis indicated different structure of soil bacteria under the birch stand compared with the others. This discrepancy resulted probably from large physiological redundancy of soil bacteria. Several bacteria species may be able to metabolize the same C substrates; therefore the bacterial communities differing in genetic structure may exhibit similar physiological profiles [31]. Another possible explanation is the fact that both methods consider only a small part of soil bacterial communities. Biolog® assay includes approximately only 1% of soil bacteria that are able to grow on the plates [32], and DGGE can detect only 1-2% of the bacterial population representing dominant species present in an environmental sample [32]. Similar to our results, Priha et al. [33] reported that Biolog® did not reveal differences in physiological abilities of soil microbial communities under the pine and birch stands despite soil microbial communities under these stands exhibited different PLFA profiles. These authors assigned different results obtained with two methods to the fact that PLFA analysis considers the whole microbial community, whereas the Biolog® test only culturable bacteria.

Conclusions

Scots pine and silver birch did not affect the C_{org} and N_t contents in the studied mine soils. However, quality of organic matter differed between these two tree species as indicated by different C_{org}-to-N_t ratio.

The soil under silver birch contained larger and more active microbial biomass than the soil under pine. Under the mixed stand, most of the gross microbial properties were intermediate between the pine and the birch stand.

The DGGE profiling indicated different composition of soil bacteria under the birch stand compared to two other stands. Differences in physiological profiles were less pronounced, probably due to functional redundancy of soil bacteria.

Acknowledgements

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