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Distribution of prokaryotic communities throughout the Chernozem profiles under different land uses for over a century

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ABSTRACT

Land use affects physical, chemical and biological properties and processes in soil. Long-term field experiments were employed to reveal changes of soil characteristics induced by land use. Using high-throughput 16S rRNA gene amplicon sequencing and quantitative PCR, comparative analyses were conducted on prokaryotic community structure in different soil diagnostic horizons of Chernozems under forest, fallow and arable land within a long-term field experiment established by V.V. Dokuchaev in 1892. Soil organic carbon (SOC) was a more sensitive and reliable indicator of changes than microbial diversity indexes. *Verrucomicrobia* changed most among different prokaryotic phyla. Long-term tillage did not result in detectable changes in α-diversity of Chernozem prokaryotic communities, except for that plow pan horizon that showed a pronounced decrease in microbial diversity. The differences in prokaryotic community structure between soil horizons were more contrasting than between land uses. Analysis of β-diversity indicated that soil microbial communities at different depths formed non-overlapping clusters of A and B horizons, while microbiomes of transitional AB horizons fall in between these two clusters. The sharp decline in α-diversity in the plow pan horizon, as well as significant differences between the communities of A and B horizons indicate that the soil microbiomes are horizon-specific.

1. Introduction

Soil is the most complex environment with greatest microbial diversity (Nannipieri et al., 2003; Torsvik and Øvreås, 2002). Bacterial and archaeal communities play essential roles in many soil processes, e.g., participating in biogeochemical cycles and maintaining soil health (Basak and Biswas, 2010; Chaparro et al., 2012; Pereira e Silva et al. 2013; van Bruggen et al., 2017). Most of soil microbes (up to 80–99%) cannot be identified and characterized by culture-dependent techniques (Amann et al., 1995), however, novel molecular approaches, such as real-time PCR and high-throughput sequencing enable to identify and quantify many uncultivable and minor species of soil microbiota (Fierer et al. 2005; Jones et al. 2009), as well as to discover the linkages between soil and its microbial community (Morales and Holben, 2011).

Soil properties are the important ecological factors that control composition and activity of soil prokaryotic communities through various endogenic physiological and biochemical processes (Upchurch et al. 2008; Gattinger et al., 2002; Smalla et al., 2001). It is well known that soil microbial communities are influenced by a wide range of ecological factors, such as pH, soil organic matter quantity and quality, plant cover, moisture availability, temperature and aeration (Eilers et al., 2012; Fierer and Jackson, 2006; Lauber et al., 2009; Rousk et al., 2010; Loeppmann et al., 2016).

Although microorganisms inhabit the whole soil profile, our knowledge on structure and diversity of soil microbial communities is mostly limited to uppermost soil horizons, while microbial communities of deeper soil horizons are not still narrowly studied (Eilers et al., 2012). According to some estimations, subsurface horizons contain up to 35–50% of the soil microbial biomass (Fierer et al., 2003; Schutz et al., 2010; van Leeuwen et al., 2017). Because of significant changes in physical and chemical soil properties with depth, it is obvious to expect strong shifts in microbial community structure from surface to sub-surface soil horizons. Therefore, it is necessary to study the full soil profile for the complete estimation of the soil microbial diversity.

There are two main approaches to study the vertical distribution of soil microbial communities. The first approach is based on nominal separation of soil layers by uniform depths, while the second one distinguishes soil diagnostic (or genetic) horizons. The first approach

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predominates in the most soil microbial ecology studies (Blume et al., 2002; Eilers et al., 2012; Fierer et al., 2003), due to its universalism and capability to estimate and compare microbial biomass in soil profiles with various vertical differentiation patterns. The second approach takes into consideration genetic formation of distinct horizons across soil profile (Rogers and Tate, 2001; Semenov et al., 2016; Will et al., 2010; Chernov et al., 2017; Kutovaya et al., 2015; van Leeuwen et al., 2017), which is used for soil diagnostics and classification (Classification and Diagnostics of Soils of the Soviet Union, 1977; IUSS Working Group WRB, 2015). The second approach is less convenient for the comparative studies of microbial communities in soils across spatial gradients. However, the differentiation of soil into distinct diagnostic horizons enables to link microbial community structure and soil-forming processes and soil properties which vary greatly in different soil horizons. Soil microbial community structure and its dominant taxa may be horizon-specific, which was shown by high-throughput sequencing of soil DNA (Will et al., 2010). In most cases, microbial diversity of upper organo-mineral horizons (A) is higher than in deeper mineral horizons (B) (Will et al., 2010). Moreover, variation between microbial communities structure in distinct horizons across single soil profile can be more prominent then variation across surface soils from a wide range of biomes (Eilers et al., 2012; Chernov et al., 2017). Thus, considering diagnostic horizons can be a prospective approach to study the vertical distribution of microbial communities throughout the soil profile.

Soils under different land-use types significantly vary in physical, chemical and biological properties, and, as a consequence, in microbial functioning and diversity (Lauber et al., 2009; Sala et al., 2000). An overwhelming number of studies have revealed effects of land use on soil microbial communities, e.g., shift in the abundances of different microbial groups depending on dominant plant species, agricultural practices, or the application of organic and mineral fertilizers (Jangid et al., 2008; Will et al., 2010; McCaig et al., 2001; Lauber et al., 2009). However, as it was mentioned above, soil microbial community responses to land-use effects were tested mostly for surface horizons.

Although the geography of soil microbial communities studies based on soil DNA high-throughput sequencing covers nearly all regions of the world, extremely diverse soils of Russia are still poorly investigated by this method (Chirak et al., 2013). At the same time, soils of European part of Russian Federation are well studied from the genetic soil science point of view, including the linkages between soil properties and soil forming factors. One of the most well-studied and famous Russian areas is “Kamennaya Steppe” nature reserve territory which represents a unique sequence of long-term field experiments established by V.V. Dokuchaev in 1892. In contrast to long-term field experiments on Rothamsted Station, whereas a wide range of molecular studies on soil microbiome were investigated (Hirsch et al., 2009; 2016; Zhalnina et al., 2015), soil microbial communities of “Kamennaya Steppe” have been extremely poorly studied.

In this study, we performed the comparative analysis of soil microbial communities structure throughout the full profiles of Chernozem soils located on the territory of “Kamennaya Steppe”. We wanted to determine the soil microbial communities shifts after more than a hundred years of being under forest, fallow and arable land. To study variation in microbial communities at different parts of soil profile, we have applied the soil diagnostic horizons approach. As microbiological parameters of soils, we estimated prokaryotes taxonomic composition, microbial diversity indexes, and the abundances of archaeal and bacterial genes by high-throughput 16S rRNA gene amplicon sequencing and quantitative real-time PCR.

2. Materials and methods

2.1. Soils and sampling sites

Soil samples were collected on the territory of agroecological station “Kamennaya Steppe” in July 2014. With a total area of 5232 ha, “Kamennaya Steppe” is located on south-west of Voronezh region on interfluvial plain between Bityug and Hoper rivers. The relief of the station is an undulating plain with mild-slope gullies and unshaped depressions. Climatic conditions are moderate continental, with cold winters and warm dry summers, and insufficient moistening. Average annual precipitation is about 420–440 mm. The average air temperature is -9.4 °C in January and −9.7 °C in February. The warmest month is June, with the average temperature of +20.1 °C. Vegetation period lasts 188 days. During winter months, soil freezes down to the depth of 60 cm.

Three full soil profiles under different land-use types were considered: 1) arable land (long-term field experiment from 1892) under winter wheat after harvesting (N 51°01′44″, E 40°43′29″); 2) fallow land (from 1882) under herbs and cereals with domination of *Festuca valesiaca*, *Bromus arvensis*, *Elytrigia repens*, *Poa arvensis*, and *Achillea millefolium* (N 51°01′51″, E 40°43′39″); 3) forest (planted in 1903) with *Quercus robur* and *Acer platanoides* as dominant species (N 51°1′41″, E 40°43′31″). Deep tillage of arable soil was annually performed. Field crop rotation included cereals (wheat, barley, maize) and tuber crops (sugar beet, sunflower). Mineral fertilizers were applied at a rate of 45–60 kg NPK ha^{-1}. Rainfall of 0.8 mm occurred nine days before soil sampling.

All three soils were classified as Typical Chernozems (Classification and Diagnostics of Soils of the Soviet Union, 1977), or Haplic Chernozems (Pachic, Clayic) (IUSS Working Group WRB 2014). The detailed description of soil profiles morphology is given in Appendix A in Supplementary materials.

Soil samples (about 100 g of each sample) were collected from the middle parts of three soil profile walls (i.e. three spatial replicates per soil diagnostic horizon) and stored then at −70 °C for further DNA extraction and chemical analyzes. The distance between the sampling points within one diagnostic horizon was about 100 cm. The total soil organic C (TOC) and total N (TN) contents were estimated by Vario MACRO Cube CN-analyzer (Elementar Analysensysteme GmbH, Germany). Soil samples for organic carbon measurements were pretreated with 0.5 M HCl to remove carbonates (Harris et al., 2001). Soil pH was measured with a potentiometer in a 1:2.5 soil/water suspension. All chemical analyzes were performed to each of three spatial replicates. Particle size distribution analysis was performed with a Laser-Particle-Sizer «Analysette 22 comfort» (FRITSCH, Germany), equipped with a low-power (2 mW) Helium-Neon laser with a wavelength of 632.8 nm as the light source.

2.2. Soil DNA extraction and purification procedure

DNA was extracted and purified from 0.25 g of each spatial replicate using PowerSoil DNA Isolation Kit (Mobio Laboratories, Solana Beach, CA, USA) according to the manufacturer’s specifications. Homogenization of the soil samples was performed using Precellys 24 (Bertin Technologies, France). Extracted DNA samples were stored in −20 °C until further analyzes.

2.3. 16S rRNA archaeal and bacterial quantification

The relative abundances of bacterial and archaeal 16S rRNA gene copies were analyzed by quantitative PCR using EvaGreen Supermix (concentrated buffer with deoxyribonucleotides, Sso7d-fusion polymerase, $MgCl_2$, EvaGreen dye and stabilizers) (Bio-Rad, Hercules, USA) and 1 μl of template DNA. Cloned fragments of *Escherichia coli* and FG-07 *Halobacterium salinarum* ribosomal operons were used to prepare standard solutions of known concentrations. Primers Eub338 / Eub518 (Lane, 1991) and arc915f/arc1059r (Yu et al., 2005) were applied for bacteria and archaea, respectively.

The reaction was carried out in iCycler (Bio-Rad, Hercules, USA) using the following protocol: 94 °C for 15 min, followed by 40 cycles of

94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s. Melting curve analysis was done for amplicon length check. Archaeal and bacterial genes copy numbers were estimated using a regression equation for each assay relating the cycle threshold (Ct) value to the known number of copies in the standards. All qPCR reactions were performed in triplicate (3 spatial replicates per horizon) .

2.4. Bar-coded pyrosequencing of bacterial and archaeal communities

The purified DNA templates were amplified with universal multiplex primers F515 (5′-GTGCCAGCMGCCGCGGTAA-3′) and R806 (5′-GGACTACVSGGGTATCTAAT-3′) (Bates et al., 2011) targeting the variable region V4 of bacterial and archaeal 16S rRNA gene. Each multiplex primer contained the adapter, 4-bp key (TCAG), 10-bp barcode and primer sequences. The expected length of the amplification product was 400 bp. Purification, pooling and pyrosequencing of the amplicons were performed with reagents according to manufacturer's instructions (Roche, Branford, USA). 16S rRNA gene pyrosequencing analysis was performed for al spatial replicates.

2.5. Processing of pyrosequencing data

16S rRNA gene sequencing data were processed in QIIME (Caporaso et al., 2010). To reduce sequencing errors, the multiplexed reads were first filtered for quality and grouped according to barcode sequences. Sequences were omitted from the analysis if they were < 200 bp, had a quality score < 25, contained uncorrectable barcodes, primers, ambiguous characters or a homopolymer length equal or greater than 8 bp. Also, all non-bacterial ribosomal sequences and chimeras were removed from the library. OTU picking was performed based on 97% sequence similarity with reference gene sequence library in Greengenes database. Determination of the microbial community structure on different taxonomic levels was performed with RDP classifier (http://rdp.cme.msu.edu/).

To estimate α-diversity, the Chao1 and Shannon diversity indexes were calculated. The efficiency of these two indexes for sequencing data treatment was shown before (Chernov et al. 2015b). The pairwise weighted Unifrac (Lozupone et al. 2011) and Bray-Curtis dissimilarity (Bray and Curtis, 1957) were used to access β-diversity patterns. The results were presented in Non-Metric Multidimensional Scaling (NMDS) using QIIME. All estimates were measured for the normalized data (normalization was carried out up to the smallest number of sequences present in the sample).

2.5. Statistics

The means of three replicates are presented in figures. A multiple *t*-test was performed to test for significant ($P < 0.05$) differences of individual microbial taxa. The differences between soil prokaryotic communities among land-use types and soil horizons were assessed by UniFrac distance-based non-parametric permutation tests for homogeneity of multivariate dispersions (PERMDISP) and variance (PERMANOVA) using QIIME.

3. Results

3.1. Soil properties depending on land use and depth

Profiles of Chernozem soils were stratified by diagnostic horizons, and morphology of each horizon was described in detail (see Appendix A in Supplementary materials). For all three studied soils, high TOC content in the A horizons (upper 70–80 cm) and its exponential decrease with depth was observed (Fig. 1; Appendix B, Table S1). In the upper humic horizons, TOC and TN contents significantly decreased from forest over fallow to arable land (8.1, 6.1 and 4.0%, correspondently). Forest and arable Chernozems characterized by a slightly acidic pH in upper horizons and the strong shift to strongly alkaline pH in the lower horizons. Chernozem under fallow had a slightly alkaline pH straight from the surface (Fig. 1; Appendix B, Table S1). All three soils characterized by the high clay (up to 49.8%) and silt (up to 53.2%) contents (Appendix B, Table S2). The A horizons of forest chernozem had much lower clay content and higher moisture content compared to fallow and arable soils. Altogether, soil moisture content at the time of sampling was not variable throughout the soil profiles, while the clay content tended to increase with soil depth (Appendix B, Table S2).

3.2. Relative quantities of bacteria and archaea estimated by quantitative real Time-PCR

The average copy numbers of 16S rRNA genes in the soil ranged between $1.0 \times 10^{10} – 5.0 \times 10^{10}$ copies g^{-1} for the upper humic horizons (A) and between $1.7 \times 10^{9} – 6.0 \times 10^{9}$ copies g^{-1} for the lower mineral horizons (B). For all studied soils, the copy numbers of 16S rRNA genes reduced with soil depth (Fig. 2A; Appendix A, Table S3).

The archaeal gene copy numbers varied from 0.67 to 11.28% of total 16S rRNA gene copy numbers depending on the soil horizon. The highest archaeal abundance (2.0×10^{9} copies g^{-1}) was detected in subsurface horizon Ah2 (15–30 cm) of Chernozem under fallow. Arable Chernozem characterized by the lowest archaeal abundance, excluding the upper horizon Ahp1 (Fig. 2A).

The highest number of bacterial 16S rRNA gene copies was detected in the upper horizon Ahp1 (0–8 cm) of arable Chernozem (4.8×10^{10} copies g^{-1}), while the amount of bacterial genes in soils under fallow and forest was up to 1.2×10^{10} copies g^{-1}. In subsurface horizons from 15 to 40 cm, the numbers of bacterial gene copies were equal for all studied soils (3×10^{10} copies g^{-1}). For arable Chernozem, the bacterial genes number reduced from surface to subsurface horizon, whereas bacterial genes numbers in forest and fallow soils were maximal in the subsurface (15–40 cm) horizons (Fig. 2B). For arable Chernozem, the bacterial gene copy number sharply reduced from 27.7 to 5.7×10^{9} copies g^{-1} from surface to Bhk horizon (40–65 cm), and did not change in lower horizons thereafter (Fig. 2B). In the deeper horizons (from 100 cm and below), the bacterial gene copy distribution pattern was similar for all studied soils.

The significant correlation with power law dependence was observed between the 16S rRNA gene copy number and TOC content in soil ($R^2 = 0.53$ for archaeal and $R^2 = 0.65$ for bacterial gene copy numbers) (Fig. 3). With low soil organic carbon availability, even a small increase in TOC content resulted in a pronounced growth of archaeal and bacterial quantities. Conversely, the changes in TOC content did not affect strongly the number of microorganisms in the C-rich (> 2%) horizons of Chernozem (Fig. 3).

3.3. Microbial community structure

In total, 144 422 sequences with an average length of 267.2 bp were obtained after quality check. At the phylum level, prokaryotic community of the studied soil samples consists mainly of *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, *Gemmatimonadetes*, *Nitrospirae*, *Planctomycetes*, *Proteobacteria*, *Verrucomicrobia* and archaeal group *Thaumarchaeota* (Fig. 4). Three bacterial phyla predominated the soil microbial communities: *Actinobacteria* (up to 55.5%), *Proteobacteria* (up to 35%) and *Verrucomicrobia* (up to 55%) (Fig. 4). Phyla *Verrucomicrobia, Bacteroidetes* and *Thaumarchaeota* had significantly ($p < 0.001$) higher rates in the A horizons, while phyla *Actinobacteria*, *Chloroflexi, Nitrospirae*, and *Planctomycetes* predominated ($p < 0.001$) the B horizons.

Verrucomicrobia was the most variable phylum depending on the horizon depth and on the land-use type. In the uppermost subsurface horizon, *Verrucomicrobia* decreased in the order forest (23.5%) > fallow (18.7%) > arable land (6.1%). The local maximum rate of *Verrucomicrobia* was observed in Chernozems under arable land

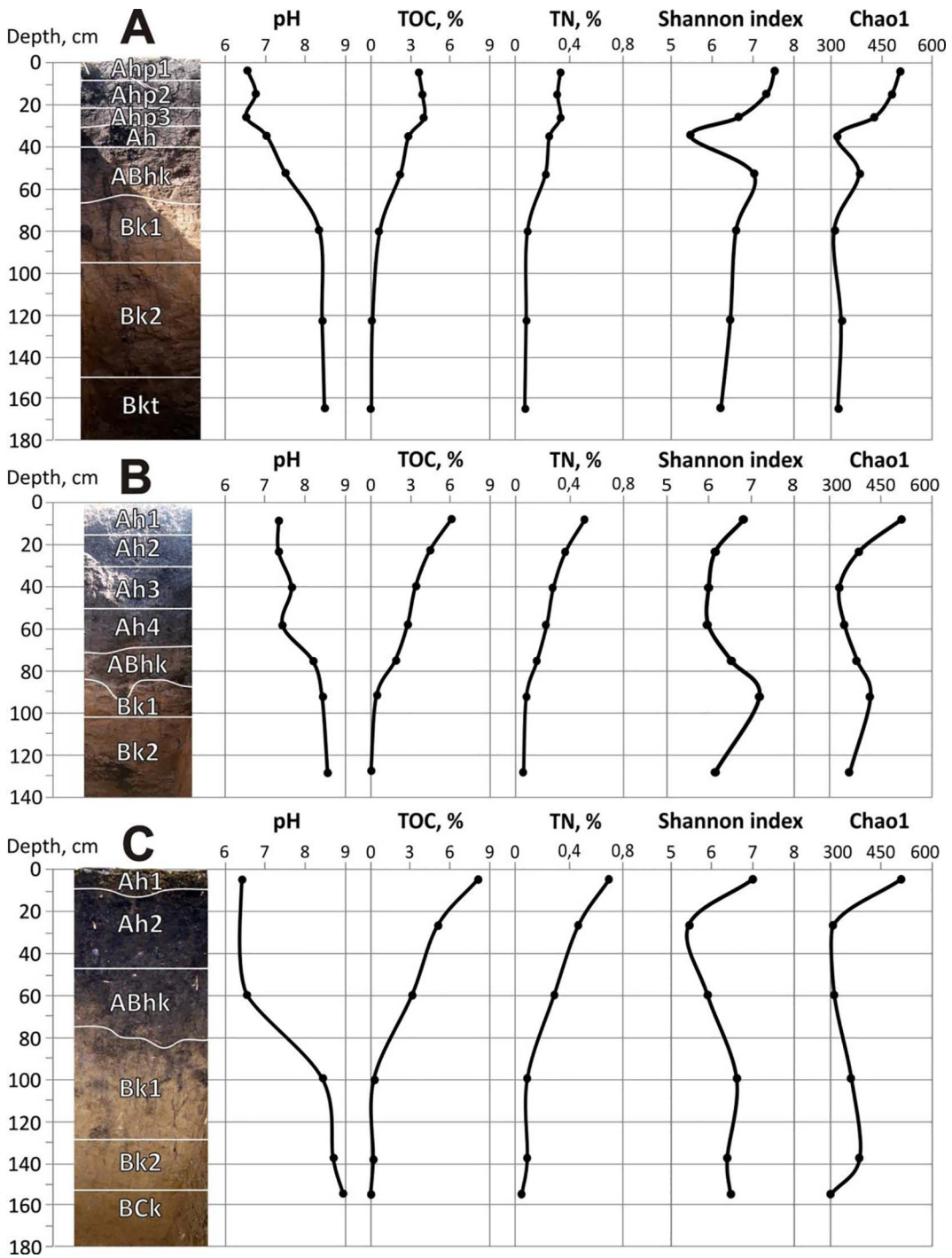

Fig. 1. Morphological, chemical and microbial α-diversity characteristics of the different soil horizons within the profiles of Chernozem soils under arable land (A), fallow land (B), and forest (C).

(22%) and mainly under forest (55%) in subsurface horizons Ahp3 and Ah2, respectively (Fig. 4). In the deeper horizons, the relative abundance of *Verrucomicrobia* in prokaryotic community was only 1–5% of total microbiome. The most dominant genus belonging to the phylum *Verrucomicrobia* was *Chthoniobacter* (up to 64.7% of all verrucomicrobial sequences).

Families *Gaiellaceae* (up to 24.5%), *Chitinophagaceae* (up to 9.9%), *Hyphomicrobiaceae* (up to 6%), and *Syntrophobacteraceae* (up to 6.9%) were among the dominant taxa in the studied soils. Family *Chitinophagaceae* was found only in the subsurface horizons, while genus *Rhodoplanes* was typical for the upper part of soil profile. The abundance of *Rhodoplanes* was lower significantly ($p < 0.001$) in arable soil compared to Chernozems under forest and fallow.

Archaeal phylum *Thaumarchaeota* relative abundance reduced from upper to deeper horizons, with local maximum in the subsurface horizons of forest and arable soils (Fig. 5). Chernozem under fallow characterized by the highest abundance of archaea (18.9–22.4%) in the upper part of soil profile (0–65 cm). In the other two soils, the highest abundances of archaea were observed in the plow pan horizon Ah (30–40 cm) of arable Chernozem (28.1%) and in ABhk horizon (45–75 cm) of soil under forest (20.1%). In general, the abundance of archaea was lower in forest soil in comparison with Chernozem under arable land and fallow.

Phylum Thaumarchaeota was represented by three genera:

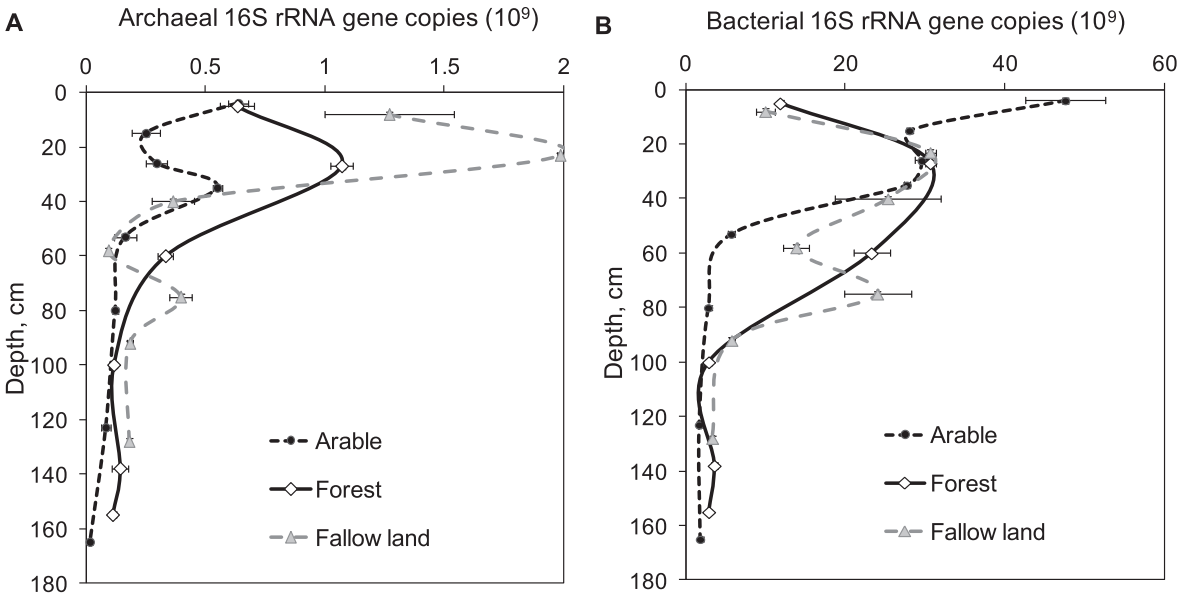

Fig. 2. The abundances of the archaeal (A) and bacterial (B) 16S rRNA gene copies throughout the soil profiles of Chernozem under three different types of land use estimated using the qPCR assays. Error bars are the standard deviations of the mean for the three replicates.

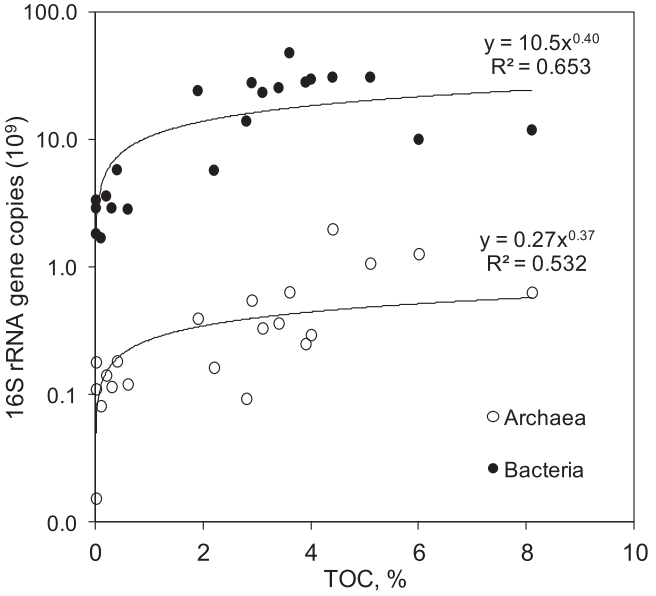

Fig. 3. Regression of the archaeal and bacterial 16S rRNA gene copies with soil organic carbon content (C_{org}) for the studied horizons of three Chernozem soils.

Nitrososphaera, *Nitrosopumilus* and *Nitrosotalea*. The dominant archaeal species was *Nitrososphaera gargensis*. *Nitrososphaera* SCA 1145, *Nitrososphaera* SCA 1170, *Nitrosopumilus* sp., *Nitrosotalea devanaterra* were also observed.

The Bacteria-to-Archaea ratios (B/A) were calculated based on data obtained by RT-PCR and high-throughput sequencing (Fig. 5). According to quantitative PCR results, archaeal gene copies ranged from 0.67 to 11.28% of all 16S rRNA gene copies, which is equivalent to B/A from 8 to 148. Based on high-throughput sequencing data, archaeal contribution into prokaryotic community was much greater in upper humic horizons (3.4–28.1%) with the B/A ratios of 2.56–28.4, while B/A ratios obtained by both RT-PCR and NGS were equal for the deeper horizons (Fig. 5).

3.4. α-diversity of the soil microbial communities

Estimation of the Shannon diversity index, number of observed OTUs, and Chao1 index indicated that microbial communities at various soil horizons hardly differed in α-diversity in most cases (Fig. 1, Appendix A in Supplementary materials). For all studied soils, the Shannon index varied from 5.5 to 7.5 (Fig. 2). The minimum α-diversity indexes were typical for the subsurface horizons at depth of 20–60 cm. A dramatic decrease of the Shannon diversity index was observed only in subsurface horizon Ah (30–40 cm) of arable Chernozem, which corresponds to the plow pan layer. The Chao1 index varied from 302 to 508 from deeper B horizons and the plow pan layer to the upper humic A horizons and showed the similar pattern as the Shannon α-diversity index (Fig. 1).

3.5. β-diversity and clustering microbial communities of A and B horizons

Discrepancy of microbiomes of A and B horizons to non-overlapping clusters was observed (Fig. 6, upper panel). The prokaryotic communities of AB horizons did not form the separate cluster, falling in between A and B horizons clusters. Microbiomes of B horizons formed small and narrow zone on the NMDS diagrams and demonstrated high degree of similarity for soils under different land uses, while the cluster of the A horizons had a wider range of values on the diagram (Fig. 6, upper panel).

Dissimilarity between prokaryotic communities of different horizons belong to soils under three types of land use has shown in the Fig. 6, lower panel. In contrast to the clear clustering by soil horizons, it was not able to cluster microbiomes of different soil horizons by land-use type.

The data on UniFrac distances were further analyzed by PERMDISP and PERMANOVA to test the differences between soil prokaryotic communities among land uses and soil horizons. When all 63 samples were considered, both tests showed similar results indicating the significant difference between soil horizons and no difference between land uses (Appendix B, Tables S4, S5). We then removed all AB and B horizons and tested only microbiomes of A horizons. It was found that the A horizons of fallow Chernozem were significantly ($p < 0.01$) different to A horizons of arable and forest soils. Surprisingly, forest and arable soils were not distinguished (Appendix B, Tables 6S, 7S).

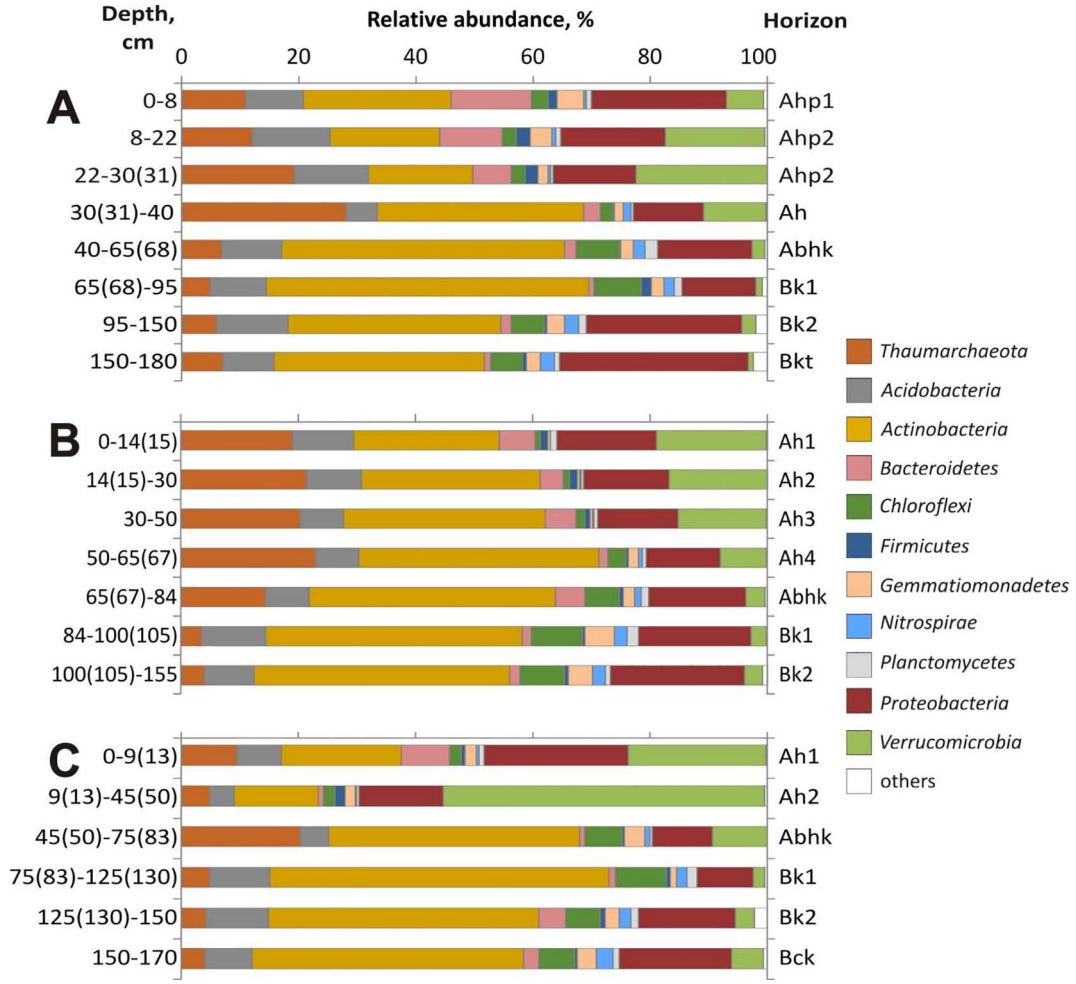

Fig. 4. Distribution of archaeal and bacterial phyla in the different soil horizons within profiles of Chernozem soils under arable land (A), fallow land (B), and forest (C).

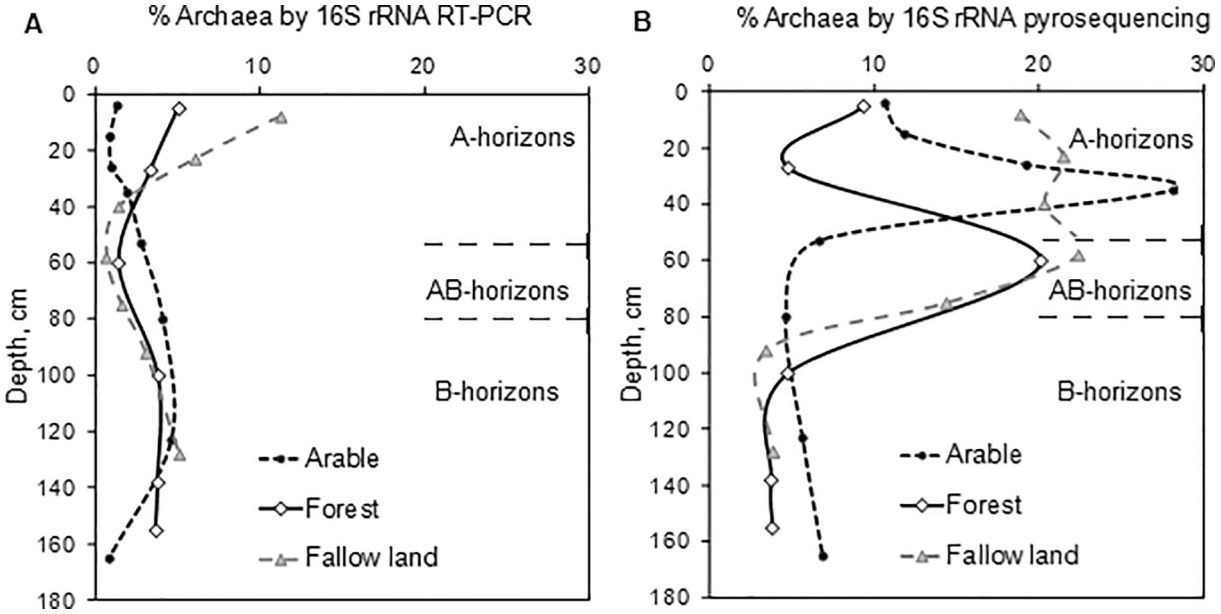

Fig. 5. Contribution of Archaea to the prokaryotic community of three Chernozem soils based on A) 16S rRNA RT-PCR, and B) 16S rRNA pyrosequencing methods.

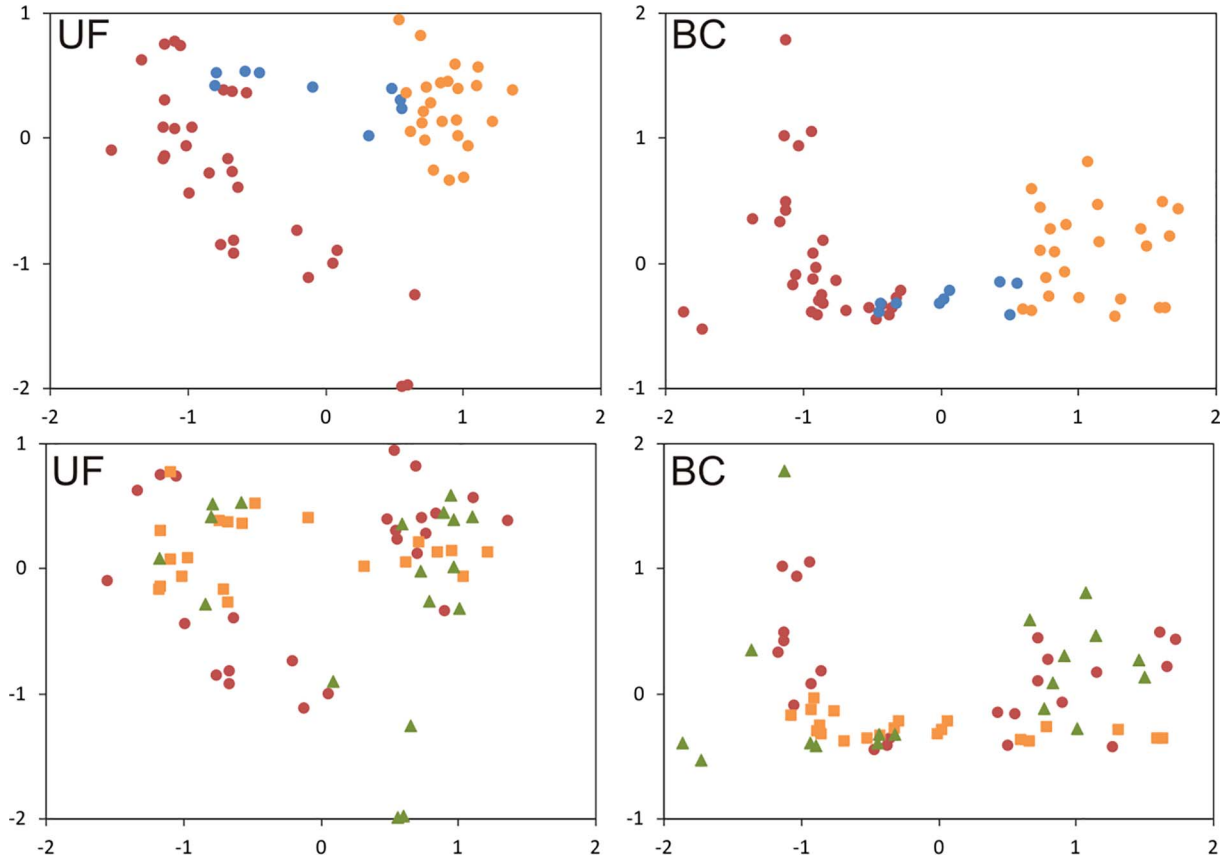

Fig. 6. Non-metric multidimensional scaling plots of soil microbial community assembly patterns using weighted UniFrac (UF) and Bray-Curtis (BC) distance matrix as related to soil horizon (upper panel) and land use type (lower panel). In upper panel, red points correspond to A-horizons, blue - AB horizons, yellow - B horizons. In lower panel, red points correspond to different horizons of arable soil, yellow - fallow soil, green - forest soil.

4. Discussion

4.1. Horizon-specific approach to study soil properties and microbial community structure

Chernozem is one of the most productive and fertile soils, with the high content of soil organic matter and nutrients and great agricultural potential. In studied soils, the SOC content was high up to 40–60 cm of depth (humic A horizons and in-between AB horizons). In the deeper B horizons, the SOC content decreased sharply to 0.1–0.6%, which is a well-known pattern of vertical distribution of organic carbon in soil (Rumpel and Kögel-Knabner, 2011). Disposition of soil diagnostic A and B horizons was clearly related to SOC distribution throughout the soil profile, which cannot be fully taken into account by nominal separation of the soil layers for uniform depths when analyzing vertical distribution of soil properties.

We expected to find strong correlation between the ribosomal genes quantity and soil organic carbon content because of their similar distribution patterns throughout the soil profile. In this study, exponential correlation between these parameters was actually observed. This means that a small increase in soil organic carbon would lead to a great raise in abundance of prokaryotes if the amount of soil organic matter is limited. Conversely, in C-rich soil horizons the prokaryotic community would not increase actively due to a further raise in organic carbon content. Thus, the organic carbon content was an important factor of the prokaryotic abundance distribution throughout the Chernozem profile and also caused the significant differences in microbial communities of A and B soil horizons. Exponential correlation between 16S rRNA copy numbers and pH values was also detected, however, this effect was caused by strong negative linear correlation between pH and organic carbon content (R^2 = 0.72). In all studied soils, the content of organic carbon decreased with depth, while pH increased. A number of studies have identified soil pH as the primary, or one of the primary, environmental variables driving soil microbial function and/or structure (Hackl et al., 2005; Fierer and Jackson, 2006). The range of soil pH values in this study may not have been as great as in other studies, or relative to the range of other site factors in this study, to display the expected relationships.

Previous studies were shown that not only organic matter content, but also soil moisture may be closely related to soil microbial community characteristics (Brockett et al., 2012). Soil moisture was found to be an important driver of both microbial community structure (Brockett et al., 2012) and overall soil microbial activity (Hackl et al., 2005). Moreover, fungal and bacterial biomass varied significantly along gradients of moisture within watersheds, and fungal biomass was highly correlated with the to long-term moisture patterns (Morris and Boerner, 1999). As far as soil moisture is a very dynamic characteristics, single measurement of microbial parameters is obviously not enough to find relationship between microbial communities and soil moisture content. Nevertheless, it has been shown before that prokaryotic communities of Chernozem at Kamennaya Steppe were characterized by seasonal fluctuations related to variation in humidity and temperature (Chernov et al., 2015a). *Acidobacteria, Bacteroidetes, Firmicutes* and *Verrucomicrobia* were revealed as taxa which were sensitive to seasonal changes (Chernov et al., 2015a). In our study, these phyla were found to be specific for the upper A horizons, while *Verrucomicrobia* varied intensively depending on the land-use type.

Using RT-PCR analysis, we determined the quantity of prokaryotic gene copies, which ranged from 1×10^{10} to 5×10^{10} 16S rRNA gene copies g^{-1} for the upper horizons of Chernozems. The obtained values were 10 times higher than the estimates of total number of prokaryotic cells (3×10^9–4×10^9 prokaryotic cells g^{-1}) by a direct microscopic

count (Manucharova et al., 2011; Lukacheva et al., 2013). The direct microscopic count method usually underestimates the number of cells due to cell adsorption on the clay minerals (Daniels, 1972; Chenu and Stotzky, 2002) which is particularly relevant for heavy clay loam Chernozems. On the other hand, RT-PCR may overestimate the quantity of 16S rRNA genes due to heterogeneity of ribosomal operons in different microorganisms (Farrelly et al., 1995; Tourova, 2003). Moreover, extracellular genes are also counted together with genes from cellular DNA using quantitative PCR (Pietramellara et al., 2009).

Estimation of the bacteria/archaea ratios revealed a big difference (up to 40 times) between the archaeal contributions into the total prokaryotic community measured by RT-PCR and high-throughput pyrosequencing methods. Quantitative PCR analysis showed that 1) archaea was a minor component of prokaryotic community, and 2) the bacteria/archaea ratios didn't change significantly with soil depth. Pyrosequencing analysis revealed the opposite trends: 1) archaea was a pronounced part of prokaryotic community, and 2) the bacteria/archaea ratios reduced sharply from A to B horizons in Chernozem. The explanation of the discrepancy between the archaeal contributions to the prokaryotic community obtained by RT-PCR and high-throughput pyrosequencing is likely to be found when considering the limitations of these methods. Both two methods are based on the amplification of the target gene regions. qPCR assays do not necessarily amplify rRNA genes belonging to all members of each targeted group (Fierer et al., 2005). Amplification produces many errors and biases due to the synthesis of artificial molecules ("Chimera", "heteroduplex"), a decrease in amplification rate efficiency during PCR cycles, difficulty in amplifying high G + C segments, and primer mismatch (Kebschull and Zador, 2015). Pyrosequencing suffers from homopolymer errors as well. The discrepancy in the B:A ratios was revealed only for A horizons, therefore these limitations could not explain strong shifts in archaeal abundance. On the other hand, two different primer systems with different specify and coverage were used for RT-PCR and pyrosequencing. The A horizons are characterized by enormous microbial diversity, and some bacterial or archaeal taxa could be underestimated.

Various studies showed both a reduce (Chernov et al., 2017) and an increase in the archaeal relative abundance with soil depth (Kemnitz et al., 2007; Eilers et al., 2012). It should be noticed that all the mentioned data was based on the DNA-approaches which do not provide information on the active bacteria and archaea. Intragenomic heterogeneity in 16S rRNA genes in different microorganisms is another difficulty in 16S rRNA studies. The amount of ribosomal genes may vary from 1 to 15 for bacteria and from 1 to 4 for archaea (Lee et al., 2009). The range of rrn genes in archaea is smaller than in bacteria that can lead to the underestimation of the archaeal relative abundance in prokaryotic communities by quantitative 16S rRNA genes analysis. Estimation of metabolically active cells by RNA-based FISH (fluorescence in situ hybridization) method revealed that the abundance of metabolically active archaeal cells increased with depth (Semenov et al., 2016). Thus, it is still not clear how the bacteria/archaea ratio varies from surface to the deeper soil horizons. The studies of distribution patterns of archaea and bacteria throughout the soil profile using both DNA- and RNA-based methods can clarify the issue.

Soil prokaryotic community structure and diversity also changed throughout the profile of Chernozems. Three prokaryotic phyla *Bacteroidetes, Thaumarchaeota* и *Verrucomicrobia* were significantly more abundant in A horizons, compared to B horizons. *Bacteroidetes* were common for the upper horizons of Chernozems, which was demonstrated for other soil types (Will et al., 2010; Eilers et al., 2012). The relative abundances of *Actinobacteria*, *Chloroflexi, Nitrospirae* and *Planctomycetes* increased with soil depth, which was also observed for Stagnosols (Will et al., 2010). The highest relative abundance of *Verrucomicrobia* was detected in the subsurface horizon (8–45 cm) of arable and forest soils, while in fallow soil no local maximum of *Verrucomicrobia* at sub-surface horizon was found. The similar pattern in the vertical distribution of *Verrucomicrobia* was previously observed in the range of studies (Bergmann et al., 2011; Eilers et al., 2012), wherein the authors detected the local peak in the relative abundance of *Verrucomicrobia* at the same "mystic" depth between 10 and 50 cm. One of the hypotheses proposed was that *Verrucomicrobia* are oligotrophic microorganisms which can grow and develop in the environments with low carbon availability (da Rocha et al., 2010; Senechkin et al., 2010; Eilers et al., 2012). However, even sub-surface horizons of Chernozems were enriched with organic carbon (4–5%) and characterized by a high biological activity, and therefore they cannot be considered as environments with low carbon availability. Moreover, a sharp decrease in the relative abundance of *Verrucomicrobia* lower 50 cm indicates that some other factors (see below) may control the distribution of this phylum throughout the soil profile.

Also, we expected to reveal lower α-diversity in the deeper horizons compared with the surface layer due to a significant decrease in organic carbon and nitrogen contents and prokaryotic abundance in mineral B horizons. However, the Shannon and Chao1 indexes did not change significantly with the soil depth, and it was not possible to separate A and B horizons by the α-diversity criteria. In a similar study, German meadow Stagnosol was characterized by stagnant overwetting of B horizons and active formation of anaerobic zones (Will et al., 2010), higher α-diversity of microbiomes of A horizons in comparison to B horizons was observed. Authors linked lower α-diversity of B horizons to the low organic carbon content, however, the anaerobic conditions unfavorable for many microorganisms may also be a reason. For the surface (aerobic) and subsurface (anaerobic) peat layers of a *Sphagnum*-dominated wetland, a significant decrease in prokaryotic α-diversity of the overwetted areas was also observed (Serkebaeva et al., 2013). Mineral B horizons of Chernozems considered in this study were not overwetted, and this may be an explanation why we did not observe any strong differences in prokaryotic α-diversity of B horizons in comparison to A horizons.

In contrast, analysis of β-diversity showed clear differentiation between A, B and even AB horizons. Microbiomes of AB horizons fall in between two clusters of microbiomes belong to A and B horizons. Similar clusterization of microbiomes from organic and mineral horizons was observed in Canadian forest soils both for bacterial and fungal communities (Hartmann et al., 2012).

Microbiomes of A horizons differed strongly from each another, and their variation was higher than that for the mineral B horizons which formed the narrow cluster in non-metric multidimensional. Similar results were obtained for a wide range of soil profiles wherein β-diversity analysis separated microbiomes of "near-surface" and "deep" horizons (Eilers et al., 2012). However, our results demonstrate that higher homogeneity of microbiomes is actually common not just for nominal "deep" layers, but namely for B soil diagnostic horizons. This tendency is in good agreement with greater heterogeneity in environmental conditions of A horizons which are more variable in pH, TOC and TN than B horizons. Thus, the ecological differences between mineral and humic soil horizons was the reason of prokaryotic community differentiation throughout the Chernozem profile.

4.2. Land-use effects on edaphic properties and microbial communities

The major distinction of the studied Chernozems was the development under different continuous land-use types within the long-term experiment from 1892 to 1902 up to now. This enabled to determine the shifts in soil properties or soil microbiological parameters. We expected to detect a falling in all measured values (TOC content, prokaryotic abundance, Shannon and Chao1 diversity indexes) for Chernozem in the order forest > fallow > arable land, and to cluster soils under forest, fallow and arable land by UniFrac and Bray-Curtis metrics. Total organic carbon content was the most reliable and sensitive indicator of land-use effects on soil status. The amount of fresh organic matter input into soil with plant residues does not compensate the soil organic carbon losses (biochemical, physical, technical)

initiated by tillage practices, which leads to a long-term decrease in soil organic carbon content in fallow and arable soils (Magdoff and Weil, 2004). The degradation of soil structure is one of the factors causing depletion of soil active organic matter and general changes in soil properties. The lower number of large soil aggregates parallels to the depletion of potentially-mineralizable soil organic matter in arable soils (Ivanova et al., 2015; Semenov et al., 2010, 2015). Moreover, application of agricultural practices alter chemical content of soil organic matter, which affects its quality and resilience (Schnitzer et al., 2006). Finally, tillage practices led to a decrease in the soil microbial biomass:TOC ratio in arable Chernozem compared to soil under forest (Semenov et al., 2018).

The bacterial and archaeal abundances estimated by 16S rRNA gene copy number analysis had a similar pattern in vertical distribution as the organic carbon content, except the upper horizons. For instance, despite the high organic carbon content in the upper A1 horizon of Chernozem under forest, the prokaryotic abundance of this horizon was much lower in comparison with the lower horizons. Conversely, a sharp increase in the prokaryotic abundance was observed in the upper horizons of arable soil. This phenomenon can be explained by a period of sampling which was performed directly after harvesting of winter wheat. In previous studies it was shown that the presence of stubble residues could be the source of additional carbon input into the soil and led to a significant increase in soil microbial biomass carbon and nitrogen (Spedding et al., 2004) and the bacterial abundance (Zhao et al., 2016).

UniFrac and Bray-Curtis methods on the 16S rRNA gene amplicon sequencing did not distinguish Chernozems under different land uses. Despite the difference in environmental conditions and organic carbon and nitrogen contents, the bacterial abundance and its distribution patterns were similar in arable and fallow soil. In contrast, the highest bacterial 16S rRNA gene copy numbers were detected in the arable soil with the lowest organic carbon content, which was likely caused by the presence of stubble residues. Archaeal 16S rRNA gene copy numbers were equal in arable and forest soils. These results contradict the previously obtained data on a pronounced decrease in metabolically active bacterial and archaeal cells in the upper tilled horizon of studied Chernozem in comparison with forest soil counted by the RNA-based FISH method (Semenov et al., 2016), as well as the lower biomass and abundance of prokaryotes in arable Chernozem in comparison with fallow one by a direct microscopic count (Polyanskaya et al., 2012).

Long-term agricultural tillage practices did not lead to a decrease in α-diversity indexes. It was not possible to distinguish soils under forest, fallow and arable land in clusters by UniFrac and Bray-Curtis metrics methods, as far as the differences in prokaryotic community structure between soil horizons were more contrasting than between three land-use types. Hirsch et al. (2009) found that microbial communities of meadow, fallow and arable soils within the long-term field experiments were not clustered when analyzing the data on 16S rRNA gene high-throughput sequencing. However, microbial communities of the same soils were successfully clustered based on the total soil RNA sequencing – i.e., analysis of the potentially active part of microbial community. Moreover, the smoothing of differences between soil prokaryotic communities under three land uses were caused by distinctive properties of Chernozem as a soil type. Chernozems of Kamennaya Steppe are highly rich with nutrients, contain the high amount of clay fraction, and are characterized by high resilience and adsorption capacity. These properties smoothes down the land-use effects and create methodology difficulties for the estimation of microbial parameters. Microbial diversity of Chernozems exceeds other soil types (Chernov et al., 2015b). According to previous studies, even long-term use of contrast doses of mineral fertilizers did not affect the taxonomic structure and microbial diversity of prokaryotic communities in Chernozem (Chernov et al., 2015a) that also indicates a high stability of microbiomes of these soils. Combining all above mentioned together, we conclude that DNA-based methods of studying soil microbial communities cannot be considered as highly-sensitive for the estimation of land-use effects on microbiological parameters of Chernozem, at least on the prokaryotic part of soil microbiome. Analysis of the metabolically active part of microbiome using transcript sequencing may be a more appropriate way to estimate the long-term impacts of different land-use types on microbial community of Chernozem.

Nevertheless, a further analysis of UniFrac distances using PERMDISP and PERMANOVA revealed that Chernozems under different land uses could also be separated in clusters when only A horizons were considered. We expected that arable and forest Chernozems would differ greatly, and the soil under fallow would take an intermediate position in between them. However, the results obtained were completely opposite to our expectations. Fallow Chernozem was significantly different to arable and forest soils, while the A horizons of the two latter were similar. Thus, two UniFrac distance-based analyzes revealed different conclusions, which means that the differences between soil microbial communities should be carefully evaluated before making an interpretation. Since only fallow Chernozem formed a separated cluster, we therefore conclude that the long-term plowing did not lead to significant changes in microbial diversity.

Also, we observed shifts in some of microbiological parameters of the studied soils depending on the land-use type. A sharp decrease in α-diversity and increase in relative archaeal abundance in subsurface horizon Ah (30–40 cm) was detected for arable Chernozem. Due to the impact of the tractor undercarriage, the pressure of the plow and other processes associated with plowing, the so-called “plow pan” layer is formed, which is characterized by a higher density, lower water permeability, microaerobic conditions, and other unfavorable physical properties (Medvedev, 2011). The main reason of those properties in arable soil is natural extenuation of soil-forming processes in the plow pan layer, which results in a poorer aggregation, and the residual soil deformation under the long-term pressure of heavy machines (Medvedev, 2011). The lower microbiological and biochemical activity, as well as weakening of soil-forming processes and an increase in thermodynamically bonded water are all common for the plow pan horizon of Chernozems (Medvedev, 2011). In Chernozems of Kamennaya Steppe, water permeability in subsurface horizon is 4–10 times lower than in the upper horizon (Tikhonravova and Perevalov 2007). Since the chemical properties of plow pan horizon were similar to those in the upper horizon, we assume that the low water permeability and the disruption of transitions between soil horizons led to a sharp decrease in microbial diversity in Ah horizon of arable Chernozem. Higher density and microaerobic conditions in plow pan horizon could also be an reason of an increase in the relative abundance of archaea which was also found for biological soil crusts (Kidron et al., 2015) and rice ecosystems (Wang et al., 2015).

Regarding the soil prokaryotic community structure, the most interesting finding was the predominance of *Verrucomicrobia* in forest Chernozem, and the lower relative abundances of this phylum in arable and fallow soils. *Verrucomicrobia* changed most intensively among different prokaryotic taxa depending on the land-use type. Although prokaryotic 16S gene copies quantification by RT-PCR analysis did not reveal the reduction in bacterial abundance, the significant decrease in metabolically active bacterial cells in the upper horizons of arable Chernozem compared to forest soil was observed by FISH method in previous study (Semenov et al., 2016), and this phenomenon may be related to the distribution of active *Verrucomicrobia*. Currently, it is still unknown, which factors control the presence and abundance of this phylum in soil. In studied Chernozems, the aerobic and saccharolytic *Chthoniobacter* (Janssen, 1998; 2006) was the dominant genera within *Verrucomicrobia* phylum. A wide range of studies showed that *Verrucomicrobia* is related to meadow ecosystems, however, we demonstrated that this phylum is abundant in soils under forest as well. Shotgun metagenomic analysis showed that the spatial distribution of *Verrucomicrobia* is associated with strong shifts in carbon dynamics (Fierer et al., 2013). As it was mentioned above, *Verrucomicrobia* domination in

the C-rich upper horizons casts doubt on the hypothesis suggesting the oligotrophic strategy of most members within this phylum. The verrucomicrobial rRNA genes abundance can be also explained by changes in soil moisture content, or some factor that is directly linked to it (Buckley and Schmidt, 2001). Metagenomic analysis and metabolic reconstruction of 'Candidatus Udaeobacter copiosus', a member within the class *Spartobacteria*, suggested that this microorganism is an aerobic heterotroph as *Chthoniobacter flavus* (Brewer et al., 2016). Moreover, 'Candidatus Udaeobacter copiosus' is also characterized by a small genome and numerous putative amino acid and vitamin auxotrophies, likely sacrificing metabolic versatility for efficiency to become dominant in the soil environment (Brewer et al., 2016).

5. Conclusions

This study demonstrates the advantages of the soil diagnostic horizons approach that enables to link microbial community structure and soil-forming processes and soil properties of the diagnostic horizons across the soil profile. Analysis of β-diversity using the pairwise weighted Unifrac and Bray-Curtis dissimilarity revealed that the microbial communities changed not only with soil depth, but clearly clustered into microbiomes of A and B horizons. The sharp decline in α-diversity of prokaryotic community in the plow pan horizon, as well as the significant differences between the communities of A and B horizons indicate the need to consider the vertical distribution of microbial communities not just for uniform depths, but for soil diagnostic horizons. Combination of next-generation sequencing with genetic soil science approach seems to be a promising way to study ecology of soil microorganisms concerning the relationship between microbial communities structure and soil properties.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.apsoil.2018.03.002.

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