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PROKARYOTIC COMMUNITIES OF TECHNOZEMS OF THE SPOIL HEAPS OF KURSK MAGNETIC ANOMALY

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❀ **Background.** Spoil heaps chronosequences are convenient models to analyze the succession of microbiome during restoration of anthropogenically disturbed landscapes. The investigation of the heavy metal content in lands with mining activity, can be used as an indicator of ecosystem recovery. **Materials and methods.** Objects were technozems of 1-year, 25- and 50-year-old embryonic soils, and control soil under forest. Quantitative polymerase chain reaction (qPCR) and NGS-sequencing of V4 region of *16S* rRNA gene were applied. **Results.** During the soil-forming process, an increase organic carbon and nitrogen, as well as a gradual increase archaeal *16S* rRNA gene copies and in the number of *Bradyrhizobiaceae*, *Blastocatellaceae*, *Xantobacteriaceae*. Although we found a number of taxa that increased during soil-forming process (*Thaumarchaeota*, *Bradyrhizobiaceae*, *Blastocatellaceae*, *Xantobacteriaceae*), technozems of different ages had a similar structure and diversity of prokaryotic communities, differing from a nature soil. Biodiversity analysis revealed that technozems generally had a similar structure and diversity of prokaryotic communities, significantly differing from the mature soil a specific clusterization of microbiomes. The HM contents and bacterial abundances remained at the same level in chronosequence. **Conclusions.** The 50 years of soil development on overburden spoil heaps is not enough for the recovery from HM contamination and restoration of soil ecosystem functioning.

❀ **Keywords:** soil microbiome; chronosequence; embryonic soils; technozems; *16S* rRNA amplicon sequencing; technogenic rock dumps.

ПРОКАРИОТНЫЕ СООБЩЕСТВА ПОЧВОГРУНТОВ ОТВАЛОВ КУРСКОЙ МАГНИТНОЙ АНОМАЛИИ

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❀ Проанализированы физико-химические параметры, растительное сообщество и структура прокариотных комплексов микробиомов однолетних (с растительным покровом и без него), 25- и 50-летних эмбриональных почв (техноземов), сформированных в районе Курской магнитной аномалии (КМА, Россия). Для анализа прокариотных сообществ использовали метод полимеразной цепной реакции в реальном времени (qPCR) и высокопроизводительное NGS-секвенирование библиотек вариабельного V4 участка генов *16S* рРНК. В процессе почвообразования, наряду с увеличением содержания органического углерода и азота, наблюдалось постепенное увеличение копий гена *16S* рРНК архей и численности бактериальных таксонов, принадлежащих к семействам *Bradyrhizobiaceae*, *Blastocatellaceae*, *Xantobacteriaceae*. Анализ биоразнообразия выявил специфическую кластеризацию микробиомов — образцы однолетних отвалов без растительности формировали отдельную группу, при этом остальные техноземы в целом имели сходную структуру и разнообразие прокариотных сообществ, значительно отличающихся от зрелой почвы. Содержание тяжелых металлов и количество бактерий в ходе почвообразования существенным образом не изменялось. Полученные результаты показывают, что пятидесяти лет недостаточно для развития почвы на отвалах вскрышных пород, установления в ней экологически безопасного уровня тяжелых металлов и восстановления функционирования почвенной экосистемы.

❀ **Ключевые слова:** почвенный микробиом; хроносерия; эмбриональные почвы; техноземы; секвенирование ампликонных библиотек гена *16S* рРНК; техногенные отвалы горных пород.

INTRODUCTION

In the past 20 years, scientists have presented extensive studies on microbiomes during primary soil-forming processes in mining sites. Interest in these subjects is due to the vast areas of disturbed lands resulting from mining operations and the identification of the key role of microorganisms in ensuring the life and development of the plant community. Microbial communities are considered as indicators of the different stages of soil restoration in technogenic landscapes [1–4]. To study the microbial succession during soil-forming processes, the method of chronosequences is used, which compares spatially separated soil differences [1, 5, 6]. This approach is very convenient and promising in the study of soil recovery from overburden soil heaps formed in mining areas mainly due to the well-known dating of the formation of heap complexes and the homogeneity of the composition of overburden spoil heaps, which serve as the basis for the formation of embryonic soil.

Ecogenesis on overburden spoil heaps in mining areas is often characterized by a high heavy metal (HM) content, affecting the succession of microorganisms during soil formation and soil restoration. HMs, such as Cd, As, Zn, Cr, and Pb, are toxic to living organisms [7, 8]. Soil contamination with HMs often leads to significant changes in the microbial diversity and structure [9, 10] or a decrease in the microbial abundance of soils [11, 12]. Li et al. [13] revealed a differentiated response of various prokaryotic groups to HM contamination. Archaea from the *Crenarchaeota* and *Euryarchaeota* groups were characterized by a positive correlation of abundance with the Cd content and showed a greater number of interactions (detected based on a greater number of links in interaction networks) with other members of the microbial community in samples with a relatively high HM level [13]. Thus, it can be assumed that archaea are more resistant to HM contamination and contribute to the adaptation of the soil microbiome to technogenic impacts.

Russian corporations account for about 40% of all disturbed lands. The Russian Federation is one of the largest iron ore manufacturers, more than half of which is produced in the Kursk Magnetic Anomaly area (KMA, Kursk region). The main parent rocks for soil formation in the area are Callovian clays covered with loess-like clays. Favorable physical and chemical parameters make loess-like substrates suitable for agriculture and land reclamation [14]. Stifeev et al. [14] demonstrated the possibility of using spoil heaps of the Mikhailovsky Mining and Processing Plant for agriculture (with preliminary placement on the surface of the dump of a humus layer of soil removed previously from the lands allocated for the mining industry).

At present, most studies on the microbial communities of technozems located in the KMA have been performed by conventional methods based on cultivation, covering only 1%–5% of the total diversity of soil microorganisms. The use of current molecular genetic methods to study the changes in the succession of microorganisms seems to be a very promising approach to assess the adaptive and evolutionary strategies of the soil microbiome during the restoration of soil ecosystems.

This study aimed to analyze the temporal dynamics of the HM content and the structure and number of prokaryotic communities in young soils (technozems) formed in the KMA region at different stages of soil formation (1, 25, and 50 years).

MATERIALS AND METHODS

Land plots of spoil heaps of loess-like loams were studied: vegetation-free rocks (LL1 and LL1b; b means barren); spoil heaps with sparse vegetation of 1 year (LL1), 25 years (LL25), and 50 years (LL50); and control soil under the woodland belt (52.2592436N, 35.3708321E). Samples were taken in triplicate from the upper soil horizon (at a depth of 0–10 cm), and variants LL25 and LL50 and control were taken at two depths, 0–5 cm (up) and 5–10 cm (down). A geobotanical description of the vegetation of the studied areas was performed, and the values of the main physical and chemical parameters in the samples were determined (as described in Ref. [14]; Tables 1 and 2).

Table 1

Description of vegetation of technozems formed on dumps of loess-like loams in the KMA

Sample (soil)	Geobotanical description
LL1 (technozem, Lithosols Technic)	Sparse vegetation; absence of arborescent layer and shrub layer. Synusiae of the coltsfoot (*Tusellago farfara*), hill-growing saltwort (*Salsola collina*), tumble-weed (*Kali tragus*), and common persicaria (*Polygonum persicaria*)
LL25 (podzolic embryonic soil)	Arborescent layer: Scotch pine (*Pinus sylvestris*), European birch (*Betula pendula*); undergrowth: Scotch pine (*P. sylvestris*), European aspen (*Populus tremula*); herbaceous layer: synusia of shorthear (*Calamagrostis canescens*), Grim-the-Collier (*Pilosella officinarum*), coltsfoot (*Tussilago farfara*), and common thistle (*Cirsium vulgare*). A significant part of the territory is covered with two types of moss
LL50 (humus accumulative embryonic soil)	Thickets of European birch (*B. pendula*) and European aspen (*P. tremula*). Forest stand formula: 5B5P; single plants: Scotch pine (*P. sylvestris*); undergrowth: English oak (*Quercus robur*), European aspen (*P. tremula*), Scotch pine (*P. sylvestris*), and European birch (*B. pendula*). The herbaceous layer is sparse, with a large number of dried (suppressed) trees [mainly Scotch pine (*P. sylvestris*), synusia bushgrass (*Calamagrostis epigeus*), and meadow fescue grass (*Festuca pratensis*); in interspaces, there is growing Grim-the-Collier (*P. officinarum*)]
Control (gray forest soil)	Arborescent layer: European birch (*B. pendula*) and Scotch pine (*P. sylvestris*), 7B3P; herbaceous vegetation characteristic of broadleaved forests: synusia of the goat's rue (*Galega gigantea*), common yarrow (*A. millefolium*), and shorthear (*C. canescens*); single plants: common chicory (*Cichorium intybus*) and sweet woodruff (*Galium odoratum*); fowl blue grass (*Poa palustris*), meadow grass (*Poa pratensis*), Grim-the-Collier (*P. officinarum*), and hair-vein agrimony (*Agrimonia pilosa*)

Table 2

Main physical and chemical characteristics of overburden spoil heaps and control soil in the KMA, %

Sample	Na_2O, %	K_2O, %	P_2O_5, mg/kg	SO_3, %	Fe_2O_3, %	CO_2, %	C_{org}, %	N_{total}, %	C:N	K_2O, mg/kg	pH_{H_2O}	pH_{KCl}
LL1b	0.84	1.95	41.7	0.34	3.85	0.08	1.69	0.05	33.8	10.1	8.61	7.46
LL1	0.98	1.83	68.3	0.16	5.11	0.09	0.62	0.04	15.5	13.2	8.06	7.03
LL25_U	0.81	1.96	16.1	0.22	5.49	2.85	1.39	0.12	11.6	24.1	8.34	7.12
LL25_D	0.74	1.95	10.8	0.19	3.55	3.3	0.91	0.03	30.3	16.4	8.62	7.38
LL50_U	0.62	1.89	46.3	0.28	4.41	0.9	1.81	0.18	10.1	24.3	8.17	7.07
LL50_D	0.87	1.91	27.1	0.19	3.64	1.5	2.01	0.13	15.5	14.3	8.37	7.17
Control_U	0.84	1.97	15.9	0.28	3.11	0.05	1.69	0.18	9.4	21.1	5.98	5.10
Control_D	0.91	2.05	13.1	0.26	3.27	0.4	1.62	0.21	7.7	14.3	5.87	4.86

DNA isolation was performed using the DNA PowerSoil® kit (MO BIO, USA), including mechanical desintegration of the soil sample using a Precellys 24 homogenizer (Bertin Technologies, France). The average DNA concentration in the sample was 50 ng/ml. Purified DNA samples (10–15 ng) were used as templates in the polymerase chain reaction (temperature profiles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s, 30 cycles in total) using Encyclo polymerase (Eurogen, Russia) and universal primers F515 and R806 to the variable region V4 of the *16S* rRNA gene. Sample preparation and sequencing were performed on Illumina MiSeq device (Illumina, USA) in accordance with the manufacturer's recommendations.

Subsequent data processing was performed using the QIIME_1.9.1 software package [15]. This process included the removal of low-quality sequences (<200 nucleotides long; quality index <25), extended homopolymer repeats, and nonbacterial and chimeric sequences. As a result, 14,312 sequences were selected; data were normalized in accordance with the number of sequences in the smallest library (4700 sequences). Sequences with more than 97% similarity were combined into operational taxonomic units (OTUs) using the *de novo* algorithm (based on the "uclust" method). From each OTU, one sequence was selected to form a representative set. The classification of the representative sequences was performed using the Bayesian rRNA classifier and the alignment of the PyNast algorithm [15]. A specially designed set, "Greengenes coreset" [15], was used for sequence alignment and taxonomy assignment.

α diversity was assessed by calculating the number of OTUs and the Faith (phylogenetic diversity) and Shannon indices. The significance of differences in the indicators of α diversity among microbiomes was assessed using the *t*-test. To analyze β diversity (assessment of the percentage of similarities/differences between microbiomes), the weighted Unifrac method was used [15]. The significance of individual taxa differences was assessed using several paired tests of the OTU frequency contingency tables. The algorithm dynamically chooses either the *G* test or Fisher's exact test and applies a correction to the Bonferroni *p*-value.

RESULTS AND DISCUSSION

Plant community analysis

In this study, 22 plant species were described (Table 1). In 1-year technozems (Lithosols Technic; the initial stages of soil formation), herbaceous forms predominated, whereas sparse forest with a predominance of birch and poplar were typical for the later stage. *Calamagrostis* and *Achillea millefolium* prevailed in the herbaceous layer in the areas of older spoil heaps, consistent with the previously described dominant species in plant communities of native soils and technozems in the KMA region [14, 17]. A minor decrease in the number of species was determined compared to previous geobotanical studies [14], which can be explained by the season during which the expedition was performed (soil was sampled in September). At that time, most plants were already at the terminal stage of vegetation. The vegetation cover on LL1 did not exceed 3%, and LL25 and LL50 were characterized by 100% vegetation cover (Table 1).

Physical and chemical properties of samples

The studied spoil heaps were characterized mainly by underdeveloped profiles of lithosols (embryonic soils) with a pronounced accumulation of organic matter in the upper soil layer only at the later stages (25 and 50 years) of succession and the control soil. For 1-year variants, soil differentiation into morphologically different horizons was not determined; therefore, they were classified as technozems. pH of the technozems was mildly alkaline (8.0), whereas pH of the control soil was weakly acidic. This was due to the high carbonate level in the rock. Fe content was high in both technozems/embryonic and background soils; there was only a mild tendency for Fe content to change over time (Table 2), which may be associated with the low mobility of iron under natural and mildly alkaline pH conditions [18].

Technozems and embryonic soils were generally characterized by higher levels of trace elements compared to the background value, whereas the HM content remained approximately the same for all stages of the chronosequences compared to the control soil (Tables 2 and 3) and background values for the region under study [21].

The levels of organic carbon and total nitrogen increase with the soil's age. There is practically

Table 3

HM content in overburden and control soil in the KMA

Sample	Ni	Cu	Zn	Ga	Pb	Rb	Sr	Y	Zr
LL1b	21	18	46	**24**	**11**	78	85	**39**	**578**
LL1	29	**21**	**52**	**22**	**15**	78	**114**	**36**	**536**
LL25_U	21	**20**	42	**20**	6	72	**122**	**33**	**532**
LL25_D	26	12	36	**20**	**11**	74	**131**	**35**	**588**
LL50_U	19	16	**46**	**20**	**10**	71	95	**33**	**521**
LL50_D	20	16	**49**	**20**	**12**	73	101	**35**	**505**
Control_U	19	19	42	**14**	**11**	78	100	**37**	**567**
Control_D	24	15	36	**20**	**18**	81	102	**37**	**589**
Background	40	20	49	10	10	84	106	30	450

Note. Values in bold surpassed the background level (according to Ref. [15]).

Table 4

Statistical values of the Mantel *R* criterion for embryonic soil samples formed on overburden soil heaps in the KMA

C_{org}	N_{total}	pH	P_{mov}	$CaCO_3$	Fe	Cu	Pb	Zn	Sr	Rb
Mantel R										
0.3	0.44	0.79	0.76	0.83	0.83	0.39	0.89	0.92	0.91	0.79
p-value										
0.0	0.01	0.01	0.00	0.003	0.004	0.02	0.003	0.00	0.001	0.00

no nitrogen in dump rocks, and it is accumulated in the soil due to symbiotic and free-living microorganisms [19]. Therefore, an increase in the nitrogen content in soils from the earliest to the latest stages of technogenic landscapes is associated with a similar accumulation of organic carbon. The carbon/nitrogen ratio (C/N) in technozems demonstrates a low enrichment of soil organic matter with nitrogen, typical for horizons containing mildly humified plant residues.

The Mantel test revealed that almost all main physical and chemical parameters have a significant effect on the structure of the microbial community (Table 4).

Number of bacteria and archaea

The highest content of 16S rRNA bacterial gene copies was found in the upper horizon (0–5 cm) of the control soil (1.29×10^{11} copies/g of soil). The number of copies of bacterial genes did not differ significantly in dump samples of different ages at a depth of 0–5 cm. The number of copies of the 16S rRNA gene was minimal in LL1b (1.03×10^{8} copies/g of soil) and 5–10 cm layer of LL25 (3.63×10^{9} copies/g of soil) compared to control and LL50 (Fig. 1).

Another tendency was found for the copy number of the 16S rRNA genes of archaea. In the control soil, the number of archaeal genes was 2.25×10^{8} and 8.51×10^{8} copies/g soil for the 0–5 and 5–10 cm layers, respectively. The number of copies of archaeal genes in the upper layers of middle-aged soil heaps was an order of magnitude higher than in the control soil. The minimum copy number of archaeal genes was detected in the LL1b sample.

Analysis of α and β diversities of loess-like loam spoil heaps

The smallest phylogenetic (Faith's index) and species (OTU quantity) diversity was revealed in LL1 and LL1b. An insignificant increase in the diversity with the age of spoil heaps (from LL1 to LL50) in the upper layer was revealed; however, the evenness (according to Shannon index) of the microbial community in the control soil was the highest (Fig. 2). The latter may indicate that each pedogenesis stage was characterized by a specific microbiome with a certain set of dominant taxa.

The multidimensional scaling of β diversity showed the grouping of samples into three main clusters, namely, soil without vegetation, control samples,

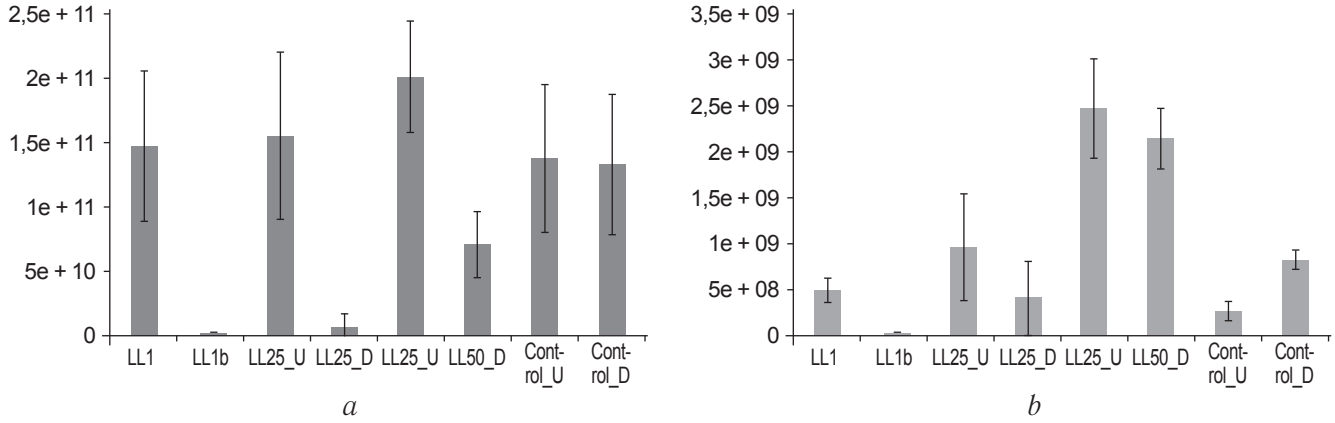


Fig. 1. Number of copies of the *16S rRNA* gene of bacteria (*a*) and archaea (*b*) in technozems (LL1 and LL1LL1b) and embryonic soils (LL25–50) of soil heaps in the KMA and the control soil (control_U and control_D)

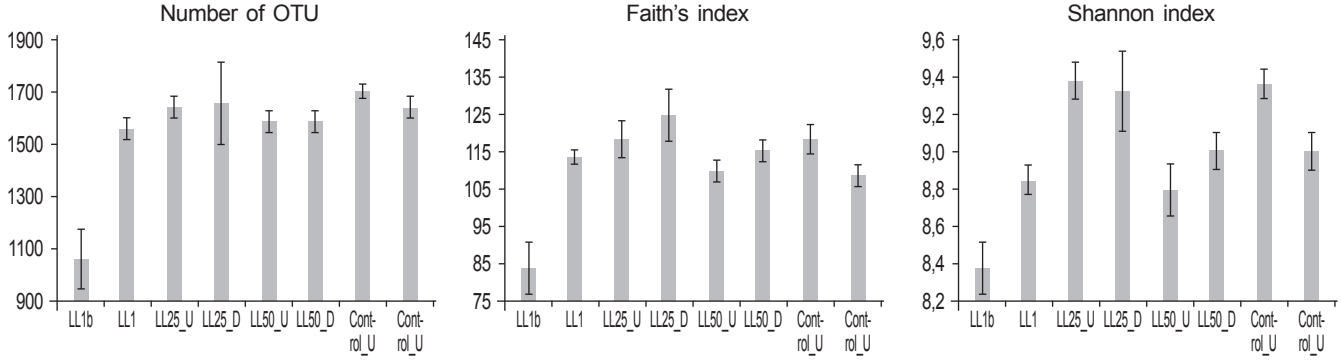


Fig. 2. Indicators of α diversity of technozems and embryonic soils of overburden soil heaps in the KMA and the control soil

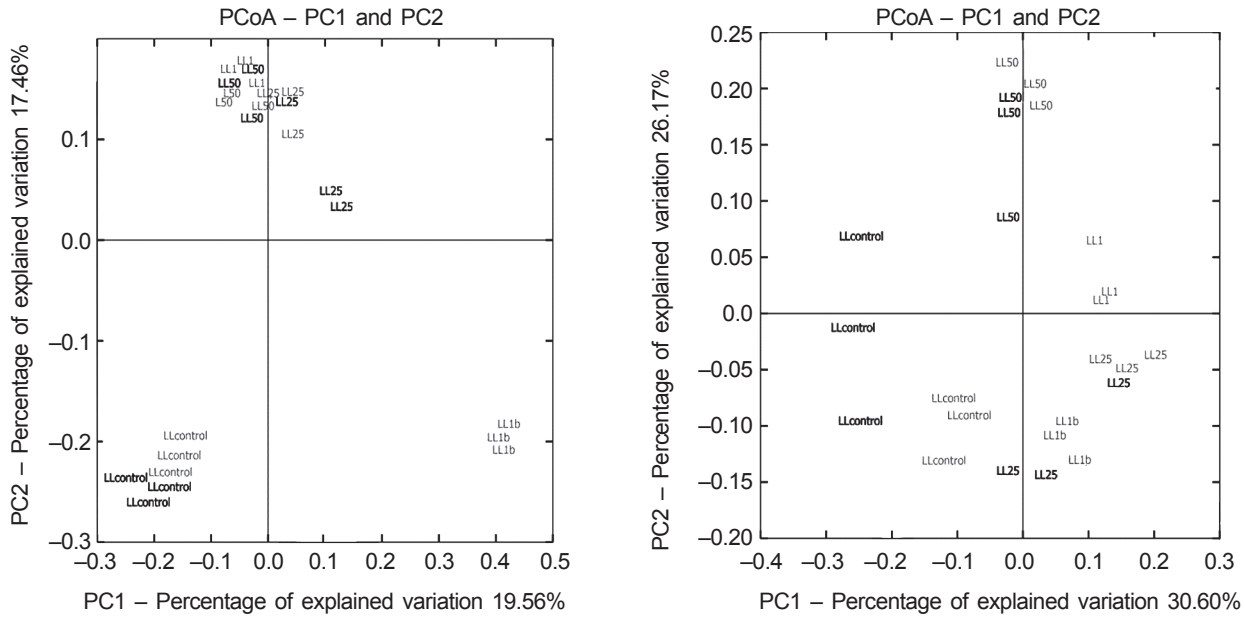
and samples of technozem with LL1, LL25, and LL50 vegetation (Fig. 3, *a* and *b*). Thus, the presence of plants was an essential factor in the formation of the soil microbiome in technogenic spoil heaps. However, the difference in microbial communities corresponding to a certain depth was noted only for younger spoil heaps (LL25). A greater heterogeneity of the lower (5–10 cm) layers, detected with age, was revealed compared to the upper (0–5 cm) layers (Fig. 3, *b*), which can be explained by the onset of soil formation in the LL25 variant.

Composition analysis of the prokaryotic community of the soil chronoseries

The analysis of the taxonomic structure of the microbiome in dump samples revealed 23 bacterial and 2 archaeal phyla. The dominant phyla were *Proteobacteria* (28.8% on average), *Bacteroidetes* (19.8%), *Actinobacteria* (18.4%), *Acidobacteria* (8.3%), *Chloroflexi* (4.3%), *Verrucomicrobia* (8.0%), and *Thaumarchaeota* (4.1%; Fig. 4). The share of other phyla did not exceed 3%.

Thaumarchaeota increased relatively in the LL50 variant (the maximum content in LL50_U was 10.2%) and the control samples. Variant LL50 was characterized by a higher Zn content, which is known to have an inhibitory effect on microorganisms [7, 10]. Thus, this element indirectly affects the number of archaea by their occupation of ecological niches occupied previously by bacteria. Previous studies revealed that many archaeal groups were characterized by an oligotrophic strategy and could win the competition for ecological niches in bacteria under energy stress conditions [20].

During soil formation in technozems, a decrease in *Gemmatimonadetes*, *Bacteroidetes*, *Gammaproteobacteria*, and *Betaproteobacteria* was noted. In mature soil, the amount of *Verrucomicrobia* was relatively increased. Some studies associated the abundance of representatives of this group with plant roots, whereas a local peak in the relative abundance of *Verrucomicrobia* is often detected at a depth of 10–50 cm, indicating the relationship of this group with the dynamics of organic carbon [21, 22].



a *b*

Fig. 3. PCoA analysis of unweighted (*a*) and weighted (*b*) Unifrac distances of microbial communities of technozems and embryonic soils of overburden soil heaps in the KMA and the control soil. The 0–5 cm layers are marked gray, and the 5–10 cm layers are black

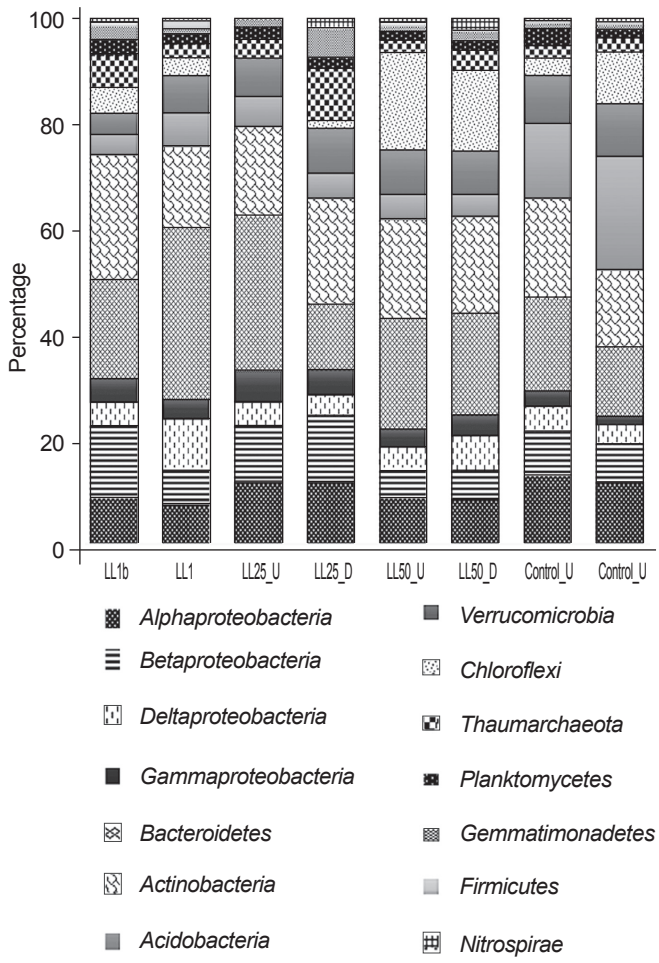


Fig. 4. Structure of the microbial communities of the soils under study at the phylum level

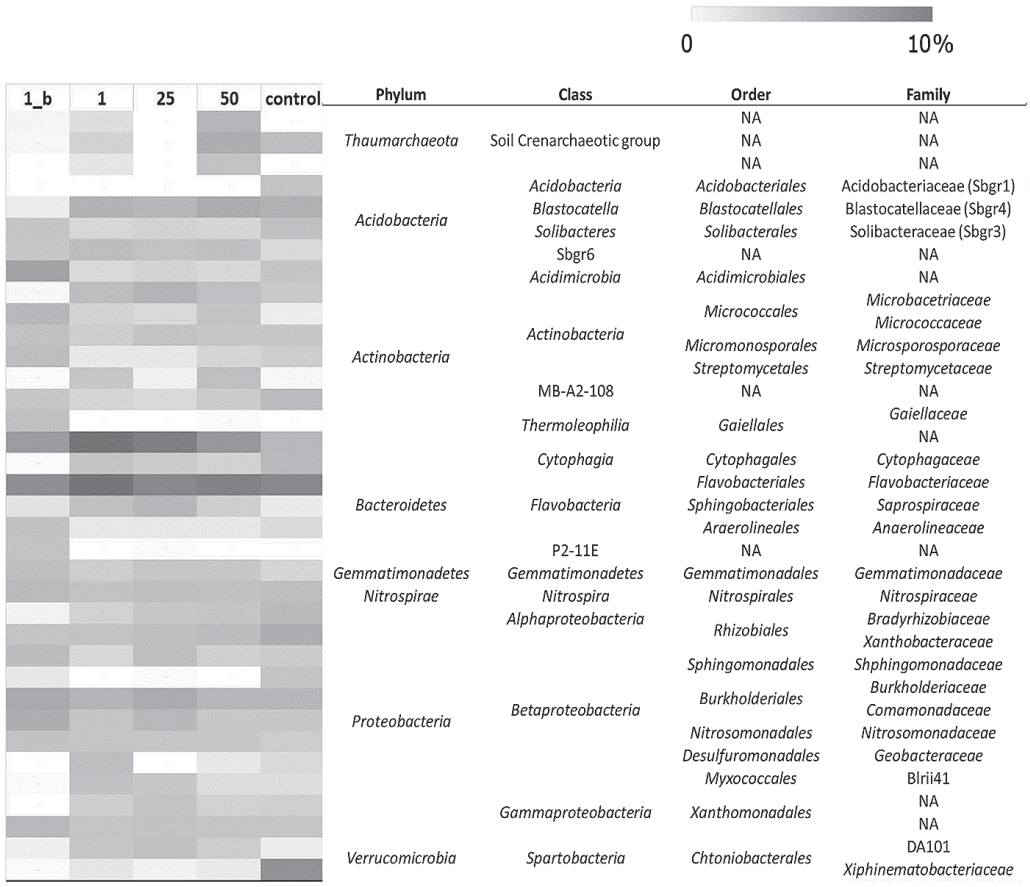


Fig. 5. Heat map of the dominant taxa associated with the soil formation stages on the soil heaps in the KMA and the control soil samples

As *Betaproteobacteria* and *Gammaproteobacteria* are often associated with the copiotrophic strategy [23], their replacement with more oligotrophic ones (*Alphaproteobacteria* and *Acidobacteria*) may indicate a transition to the climax pedogenesis stage. Bacteria belonging to the phylum *Bacteroidetes* are often involved in the mineralization processes of plant residues and considered as typical copiotrophs [23], explaining the decrease in their abundance in the lower (5–10 cm) soil layers.

Spoil heaps without vegetation (LL1 and LL1b) were characterized by a specific microbial composition. The relatively high content of SO_3^{2-} and Fe^{2+} ions can explain the appearance of chemolithoautotrophs, which are sulfur-reducing agents (e.g., *Desulfurellaceae*). In this variant, *Acidobacteria* was the lowest, and the relative content of *Actinobacteria* was increased. The latter are mainly represented by *Actinomycetes*, *Streptomycetaceae,* and *Micromonosporaceae* (Fig. 5), which can form spores and live under unfavorable conditions with a low organic carbon content and moisture. *Nitrosomonadales* (*Betaproteobacteria*) and *Sphingomonadales* (*Alphaproteobacteria*) were the dominant *Proteobacteria*. In the control samples, the proportion of *Rhizobiales* (*Alphaproteobacteria*) and *Burkholderiales* (*Betaproteobacteria*) increased. *Burkholderiales* are characterized by their wide adaptability to the environment and ability to use various substrates. The gradual increase in other typical rhizosphere microorganisms (e.g., *Bradyrhizobiaceae* and *Xanthobacteriaceae*) with soil age may suggest a possible role of these bacteria in soil restoration and specify a certain pedogenesis stage. *Bradyrhizobiaceae* were identified as part of the core component of all types of Russian soils [24]; therefore, this family of bacteria can be an indirect indicator of the soil ecosystems' climax stage.

CONCLUSION

This study compared the temporal dynamics of HM content and the structure and number of pro-

karyotic communities in technozems formed in iron ore mining areas in the KMA region at different stages of soil ecosystem restoration. A gradual increase in organic carbon and total nitrogen in soils and an abundance of archaea were noted. During soil-forming processes in technozems, a decrease in *Gammaproteobacteria, Betaproteobacteria, Gemmatimonadetes,* and *Bacteroidetes* was noted. In contrast, several taxa (*Thaumarchaeota, Bradyrhizobiaceae, Blastocatellaceae, and Xantobacteriaceae*) gradually increased in numbers. High Zn concentrations indirectly affect the number of archaea, contributing apparently to their occupation of ecological niches occupied previously by bacteria. Technozems of different ages had a similar structure and diversity of prokaryotic communities, significantly different from mature soil. These results showed that HM contamination has a long-term destructive effect on soil microbial communities. After 50 years, no significant decrease in soil contamination with HM and restoration of the soil ecosystem function was revealed on overburden spoil heaps.

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