

Research Article

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Soil microbiome of different-aged stages of self-restoration of ecosystems on the mining heaps of limestone quarry (Elizavetino, Leningrad region)

<https://doi.org/10.1515/opag-2020-0207>

received August 19, 2020; accepted September 14, 2020

Abstract: Soil microbiome plays an important role in soil forming process as well as soil functioning. This is especially relevant for initial stages of soil regeneration after strong anthropogenic impact (i.e., in quarrying complexes). The study of soil microbiome in such areas is crucial for both understanding the forces driving soil formation and optimization of the reclamation techniques. This study is aimed to investigate the soil microbiome of different ages of soil formation on the heaps of limestone mines in conditions of boreal climate of North-West of Russia. Soil microbiome analysis was performed using high-throughput sequencing. Soils of the investigated sites were predominantly Rhendzic Leptosols. The initial set (2 years) of OTUs in the entire period of soil formation remains unchanged, accumulating more than 98% of the total microbiome in each time point. Analysis of beta-diversity (weighted and unweighted) demonstrated a clear differentiation of microbiomes of all time

points, which suggests that underlying taxonomic structure variations can be attributed to variation of the minor taxa set composition. The most significant differences occur in *Proteobacteria*, *Chloroflexi*, *Acidobacteria*, and *Actinobacteria*, but majority of these phyla have different sets of OTUs demonstrating opposite trends. Generally time positively correlate with microbiome of carbonate soils in all cases of benchmark and newly formed soils is familiar because of the uniform zonal environmental conditions in geochemically specific landscape of Izhora upland.

Keywords: microbiome, soil, self-restoration, high-throughput sequencing, North-West Russia

1 Introduction

Soil microbiomes play an important role in soil functioning and implementation of key ecosystems services (Sprocati et al. 2014; Abiraami et al. 2019; Gorzelak et al. 2020). Nowadays, the possibilities of new generation sequencing allow to receive huge amounts of data on taxonomical and functional diversity of soil microbiome (Persina et al. 2019). These data can be used for management of microbiological drivers of soil formation. Key soil forming processes are connected with activity of soil microbiome directly or indirectly. This is especially important for the very initial stages of soil regeneration after strong anthropogenic impact (Urbanová et al. 2011; Sprocati et al. 2014). That is why an accumulation of the data on soil microbiome in various postanthropogenic and technogenic environments is quite useful for creation of fact-based databases for environmental engineers and decision makers in the field of soil and vegetation cover restoration (Dmitrakova et al. 2018a, 2018b). At the same time, we have now more raw and processed data on microbiome of zonal mature soils (Taş et al. 2018) than on microbial communities of abandoned or reclaimed lands, recently involved to open-cut mining (Urbanová et al. 2011). It is quite important to understand the structure of microbial

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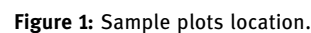
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2 Materials and methods

The study sites are located in the central part of Leningrad region (North West of Russia, Figure 1), on Izhorskaya



upland (another name – Odrovician plateau). This is an upland which has been used for intensive agricultural practices in time of Sweden's presence in this area due to high fertility of soils with high percentages of carbonates – Rhendzic Leptosols (FAO 2014). Thus, this region was intensively anthropogenically and industrially affected. In the beginning of the nineteenth century, intensive mining of limestones was started with the aim of supplying Saint-Petersburg city with limestones for buildings and facing stone. One of the most ancient limestone quarries in Lenin-grad region is located in Pudost settlement and it was designated for limestone mining for Kazanskiy Cathedral construction and facing. Elizavetino quarry is the second largest and oldest mine in Izhorskaya upland. The age of this quarry is about 200–220 years, but soils of the heaps are younger, because of many replacement materials of heaps accumulated inside the quarry. There are numerous similar quarries, located in the west direction, till the border with Estonia (Kimeklis et al. 2020; Dmitrakova et al. 2018b). All these quarries have the chronoserries of soil formation of various ages. The study sites of Elizavetino quarry are represented by the following ages: 2, 26, and 70 years of self-restoration of abandoned spoil heaps and one profile of benchmark soil of climatic stage of ecosystem development. Thus, we have classical soil chronoserries; presented by 2 years old Lithostrat, or soils with only initial features of organic matter accumulation, 26-years old Rhendzic Leptosol with well-developed A horizon in top-soil, and 70 years old Rhendzic Leptosol with well-expressed profiles, containing O-A-AC-C horizons with high amount of lime fine earth. The vegetation cover of the very initial stage of soil formation is presented by very sparse shrubs of willow (*Salix* sp.), very few seedlings of poplar (*Populus alba*), and few representatives of the Leguminosae family. The stage of 2 years is presented by

following herbal species: *Fragaria vesca*, *Calamagrostis officinales*, *Vicia cracca*, *Tussilago farfara*, *Melilotus albus*, and *Gallium album*. The tree species are presented mainly by alder (*Alnus incana*) and willow (*Salix caprea*) with some admixture of apple tree (*Malus ideaus*). At the stage of 70 years, the developed rare-stand forest is composed of willow, alder, poplar, rowan (*Sorbus aucuparia*), aspen (*Populus tremula*) with mixed and rich herb stand, and essential part of bryophyta. The benchmark soil is located under the zonal developed mature deciduous-coniferous (*Picea abies*, *Tilia cordata*, *Acer platanoides*) forest with prevalence of nemoral species (e.g., *Asarum europaeum* and numerous ephemeroids). This soil can be classified as Rhendzic Leptosol, formed on derivatives of limestones.

2.2 Laboratory analyses

Soil samples for top horizons were sampled into the plastic bags and frozen in the field; other sample types were taken from every soil horizon with the aim to characterize better soil profile development rate and vertical soil profile structure. These unfrozen samples were transported to the laboratory, grounded, and passed through 2 mm sieve.

The particle size distribution was determined using the Kaczynski sedimentation method with pyrophosphate peptization of microaggregates (Rastvorova 1983), the organic carbon content was determined by C–N–H analyzer (EA3028-HT, Research Park of Saint-Petersburg State University), and the pH of the aqueous extract was determined potentiometrically in a soil:water ratio of 1:2.5. The concentration of mobile phosphorus and potassium compounds was measured as per Kirsanov in TsINAO modification, exchangeable ammonium by TsINAO method (GOST 2011), and nitrates ionometrically (GOST 1985, 1986).

DNA was extracted in 6 replicates for each site from 0.5 g of soil using the NucleoSpin® Soil Kit (Macherey-Nagel GmbH & Co. KG, Germany) using a combination of SL1 + SX buffers. Homogenization of soil samples was performed on a Precellys 24 device (Bertin Technologies, France). The purity and amount of DNA in the preparation was assayed electrophoretically in 1% agarose with $0.5 \times$ TAE buffer (DNA concentration in a sample averaged 50 ng/ml). Purified DNA was used for quantitative PCR (qPCR) and preparation of amplicon libraries as per the instructions of the sequencing protocol (Illumina, Inc., USA).

To estimate the quantity of bacteria in the soil samples, quantitative qPCR was performed with the following primer pairs for the 16S rDNA fragment – EUB338 (5'-ACT

CCTACGGGAGGCAGCAG-3') (Lane 1991) and EUB518 (5'-ATTACCGCGGCTGCTGG-3') (Muyzer et al. 1993). qPCRmix-HS SYBR kit (Eurogen, Russia) was used to prepare the reaction mixture according to the manufacturer's instructions. The standards were the series of 10-fold dilutions of fragments of the 16S rRNA gene of *E. coli*. Each sample of the PCR mixture, including standards, was analyzed in triplicate. The measurements were carried out on a CFX96 thermocycler (Bio-Rad, Germany) according to the following protocol: 3 min at 95°C, 20 s at 95°C, 20 s at 50°C, and 20 s at 72°C (40 cycles). For replicates of both PCR and different DNA samples in a site, the mean values (M) and standard errors of the mean (\pm SEM) were calculated. After processing, the results were expressed as the number of ribosomal operons per 1 g soil. Significance of mean differences was assessed by Student test with $p < 0.05$.

In preparing libraries of 16S rRNA gene fragments for each soil DNA sample, PCR was performed with universal primers for the variable region V4, the F515 5'-GTGCCAG CMGCCGCGGTAA-3' and R806 5'-GGACTACVSGG-GTATC TAAT-3' (Bates et al. 2011) (T100 thermocycler, Bio-Rad, Germany), according to the following protocol: 3 min at 95°C, 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C (35 cycles). Sequencing and primary processing of data were performed (an Illumina MiSeq instrument (Illumina, Inc., USA)) in the ARRIAM Genomic Technologies and Cell Biology Center for Collective Use of Research Equipment. Sequenced fragments of the 16S rRNA gene were processed using the Trimmomatic (Bolger et al. 2014) and QIIME (Caporaso et al. 2010) software according to the following process. All sequences were trimmed, paired-end reads were assembled, and the quality of nucleotide sequences was checked. All nonbacterial and chimeric sequences were removed, and data were normalized. Sequences with more than 97% similarity were combined into operational taxonomic units (OTUs) using the de novo UCLUST-based algorithm. One sequence was selected from each OTU to compile a set of representative sequences. The representative sequences were classified using RDP naïve Bayesian rRNA Classifier program, and then aligned according to PyNast algorithm, using the SILVA database as a matrix.

For a comparative analysis of communities, the indices of alpha- and beta-diversity were calculated. Alpha-diversity was evaluated using species richness indices (the number of OTUs in the sample (Observed)), Faith's phylogenetic diversity index (PD), and the Shannon and Simpson indexes of diversity (Faith, 1992).

The significance of differences between microbiomes by alpha-diversity indices was evaluated using Mann-Whitney test. To determine beta-diversity, weighted and

unweighted unifracs methods were used. The results were analyzed using multivariate statistics (analysis of the principal coordinates with R analysis). A Mantel test (Pearson correlation, 100 permutations) for combined replications was performed with QIIME to assess the correlation of bacterial community composition with concentration of basic soil macroelements (Mantel 1967). To assess the significance of differences between abundance of individual taxa in different soils, DeSEQ2 library (Love *et al.* 2014) was used.

The paper presents the mean values of indicators (M) and their standard errors (\pm SEM). The revealed differences were considered statistically significant at $p < 0.05$.

Ethical approval: The conducted research is not related to either human or animal use.

3 Results and discussion

3.1 Soil development and chemical features

Soils, formed on the bottom of quarry, represent not a fast mode of initial soil development due to the fact that they are formed on the carbonate containing materials. In such cases, the speed of soil evolution is lesser than that on acid and weathered parent materials (Emmer 1995; Kurách *et al.* 2012). Normally, in conditions of Russian North-West, initial soil development is a fast process, and 70 years are enough for formation of embryopodzol on the sandy textured heaps of former quarries (Abakumov *et al.* 2010). This also has been confirmed by Mokma *et al.* (2004) for various climatic belts. At the same time, former limestone quarries indicate very low speed of soil development and differentiation into horizons (Dmitrakova *et al.* 2018b; Kimeklis *et al.* 2020); the same is known for heaps of clays and lime clays of Czech Republic (Kurách *et al.* 2012). Even in conditions of humid climate with high precipitation rate, weathering process on carbonate containing materials is not very intensive due to buffering activity of alkaline materials of the fine earth (Dmitrakova *et al.* 2018b). Thus, the soils developed on such substrates do not show evidence of strong profile differentiation into horizons (Figure 2).

Data on soil chemical composition are provided in Table 1. These data illustrate that soils investigated are typical representatives of Calcaric soils in the initial or primitive branch of national soil classification (2004). These soils are alkaline, and the degree of alkalinity

decreases with the age as well as carbonate content. This is quite typical for different-aged soils of different-aged ecosystems of Ordovician plateau (Blagovidov 1946; Gagarina *et al.* 1981; Gagarina 1996). Decreasing of carbonate content during leaching and weathering results in decreasing of skeletal fraction content and increasing of the fine earth percentages which well corresponds with data of Abakumov *et al.* (2005).

3.2 Soil microbiome

Data on soil microbiomes are presented on Figures 3–6. The distribution of copy number of 16S rRNA operons per 1 g of soils on the different age stages of soil formation (Table 2) shows the increasing trend within the age row. This parameter indicated the bulk level of biologization of substrate from the initial stage of soil formation till the climax stage, e.g., benchmark ecosystems. These data are in good correspondence with metagenomics data, obtained previously (Urbanová *et al.* 2011).

The analysis of the microbiome dynamics in the course of soil formation has shown several important features. As expected, the total sum of bacteria is constantly increasing through 4 soil ages starting with very low 10^7 and ending with 10^9 at the “mature” soil benchmark site (Table 2). This is in complete accordance with the soil carbon/nitrogen accumulation which is clear mark of the soil formation where bacteria are one of the most important players. But something is surprising: as it is evident from the Figure 3, the taxonomic composition (on the phylum level) remains almost the same through all 200-years process of the soil formation. Even the initial OTU set of the first age (2 years) remains unchanged all the history of the soil formation: the fraction of the total microbiome contained in the initial set of OTUs in all soil samples corresponding to 26, 70, and benchmark soil is about 98% in all cases. The alpha-diversity indices describing richness, evenness, and phylogenetic diversity also vary in a very narrow range (Figure 4), and according to the statistical tests, have no significant difference between soil ages. But, behind a stable picture of the alpha-diversity, a real dynamic process can be detected based on the weighted and unweighted beta-diversity analyses (Figure 5). From the data presented, it is clear that mechanisms of the soil microbiome evolution in course of the soil formation include both changing the taxa set (unweighted unifracs) and their abundances (weighted unifracs). Interestingly, both ordinations demonstrate the close similarity between soil

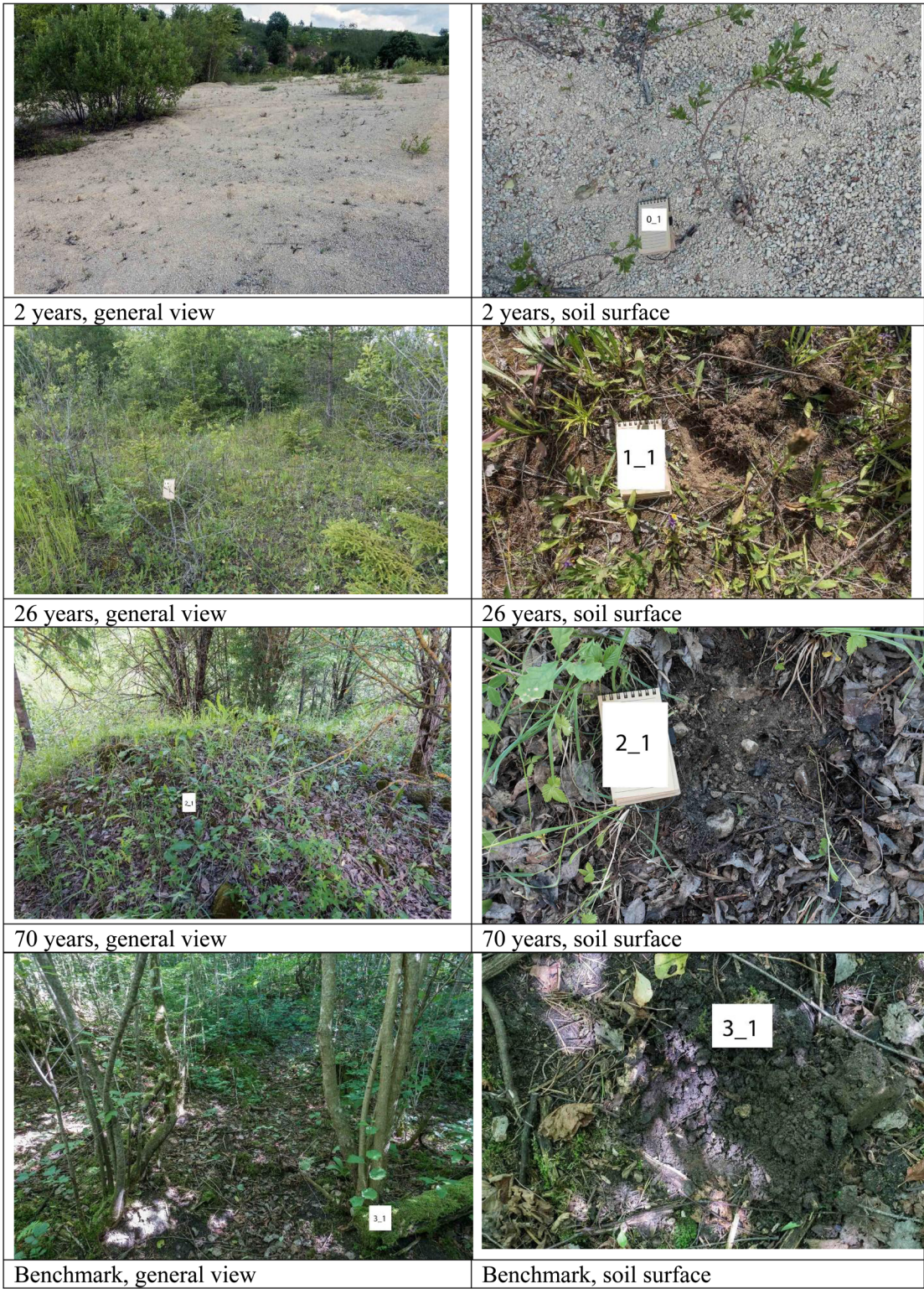


Figure 2: Soils of different stages of chronoseris.

communities of 26 and 70 years samples and separate benchmark). These data taken together demonstrate location of the initial and final points (2 years and that the evolutionary rate of the soil microbial

Table 1: Chemical properties of topsoil humus organo-mineral layers

Sample code	TOC, %	N, %	C/N	pH H ₂ O	CaCO ₃ , %	Skeletal fraction, %	Fine earth, %	Clay particles, <0.01 mm, %
2	0.30	0.04	7.5	8.20	87.4	58	42	12
26	2.02	0.20	10.1	7.80	78.9	47	53	20
70	2.20	0.20	11.0	7.70	71.0	42	58	27
Benchmark	2.22	0.15	14.8	7.30	67.1	34	66	39

community is relatively high in the first years of the soil formation and has trends to a slowdown in the course of further evolution.

There are some important details about weighted and unweighted beta-diversity metrics. Based on the above-mentioned finding that about 98% of the total communities of all soil samples of all soil ages belong to the initial set of OTUs detected in the 2-years old samples, one can propose that the dynamics of the taxonomic structure detected in the unweighted Unifrac could be attributed only to the minor prokaryotic taxa. Contrary to the unweighted metric, the difference between the soil microbial communities evident from the weighted unifrac ordination can be attributed to the taxa representing an essential part of the microbial community.

This is evidenced by both clear separation of the microbial communities of different ages and the high level of the explained variabilities (axis 1 and axis 2) which are twice the unweighted values.

For revealing active taxa (which have positive or negative abundance changing), we use DeSEQ2 algorithm. By beta-diversity, we can see nonlinear changes in communities: two clusters (22 and 70 years soils) are, in fact, really close. So we perform DeSEQ2 analysis only for two pairs: 2–26 years samples and 26–200, respectively.

According to DeSEQ2 comparison, most significant differences occur in *Proteobacteria*, *Chloroflexi*, *Acidobacteria*, and *Actinobacteria*. Those phyla contain significant amounts from all active communities. Meanwhile, many OTUs from

**Figure 3:** Taxonomy composition of soil microbiome.

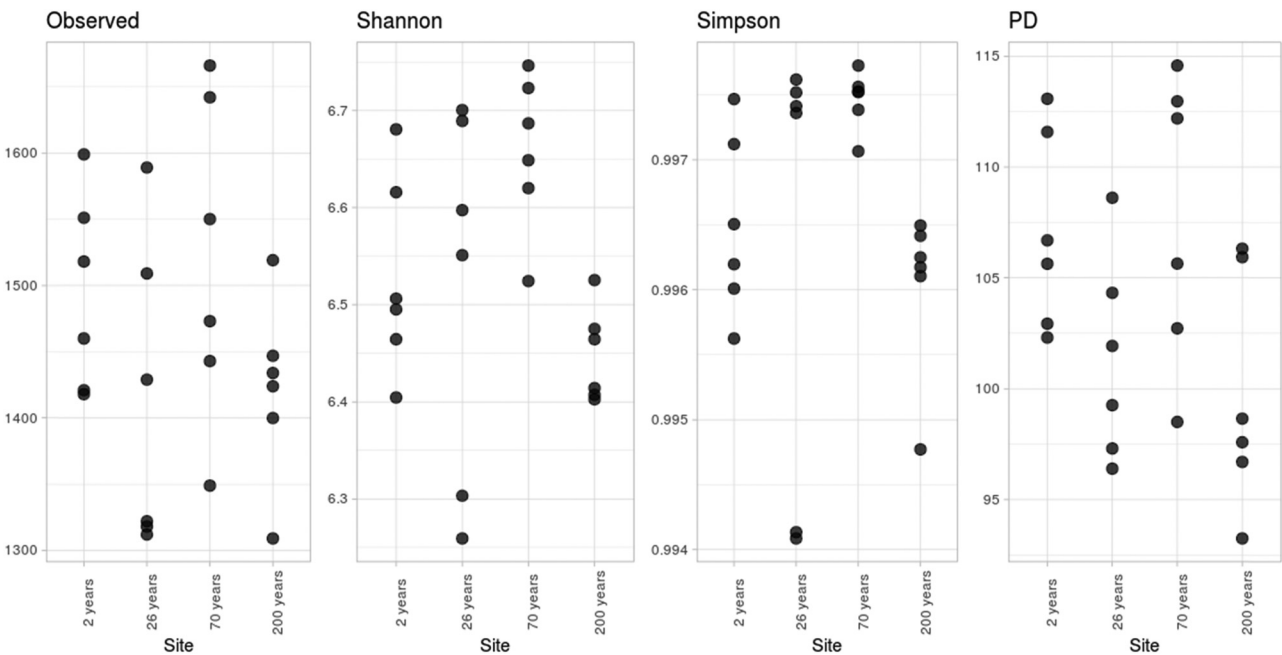


Figure 4: Alpha-diversity metrics.

this phylum show different patterns in pair comparison: there are decreasing OTUs alongside increase in the same phyla (Figure 6). Several phyla demonstrate a low number of active OTUs: they are *Firmicutes*, *Platescibacteria*, *Thaumarchaeota*, and *Verrucomicrobia*. Most intensive

increasing OTUs in comparisons belong to *Cyanobacteria* and *Actinobacteria*, followed by *Deinococcus-Thermus* (up to 8 times more in 200 years soil) and *Nitrospirae* (up to 6 times in 2 year soil). From the total 7211 OTUs in all dataset, 59 OTUs have significant changes in first comparison

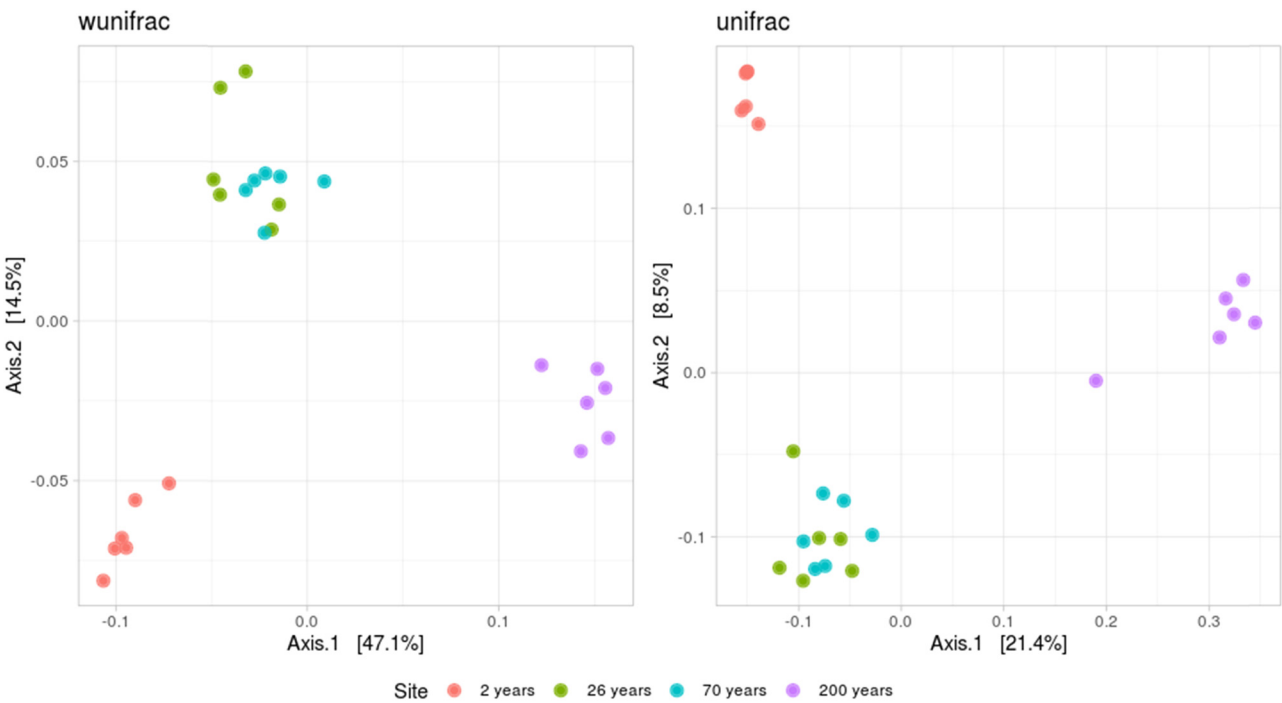


Figure 5: Weighted (1) and unweighted (2) Unifrac ordinations of the soil prokaryotic communities.

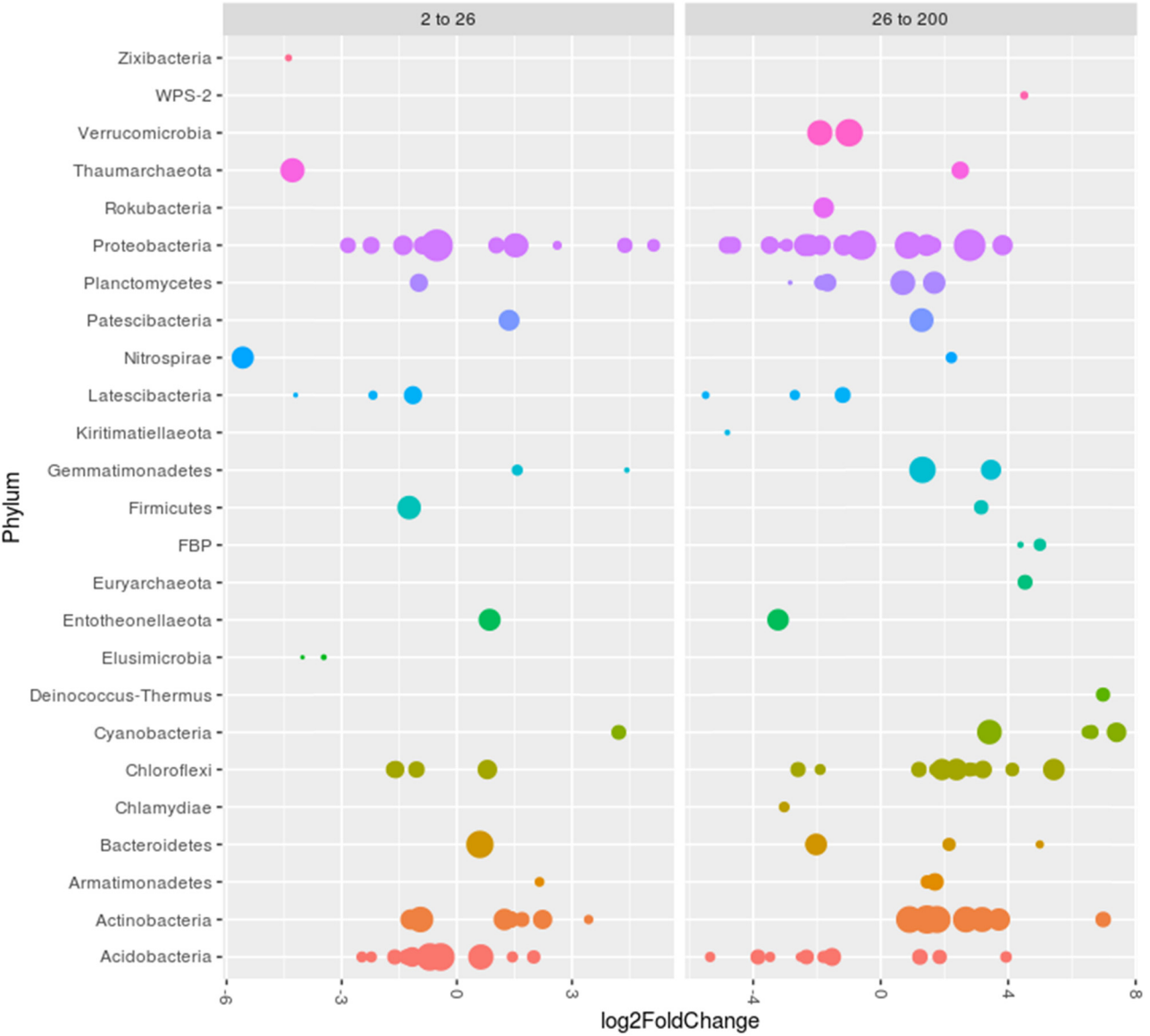


Figure 6: Changes in abundances of different OTUs at phyla level in soil comparisons of different ages.

Table 2: Bacterial abundance in study sites assessed by real-time PCR. Numbers express means with standard errors for the amount of copies of ribosomal operons per 1 g of soil. Differing superscript letters (a–c) indicate significant differences between sampling sites ($p < 0.05$)

Site	Age	Copies of ribosomal operons per 1 g soil
0	2 years	$(2.65 \times 10^7 \pm 1.13 \times 10^7)^a$
1	26 years	$(5.99 \times 10^8 \pm 8.56 \times 10^7)^b$
2	70 years	$(7.77 \times 10^8 \pm 1.13 \times 10^8)^{bc}$
3	Benchmark	$(9.27 \times 10^8 \pm 1.07 \times 10^8)^c$

(2 to 26) and 94 in second. In both comparisons, the number of “active OTUs” is 125. Number of reads from this “active

OTUs” component variates from 1,611 to 2,009, while the total amount of reads in all samples was 10,816. First 40 most abundant “active OTUs” are shown in a supplementary table (bold are groups, where difference is significant)

4 Conclusions

1. Soil chronosequences of geochemically specific environments of Izhora upland are different from those formed in postglacial environments of Russian North-West. This is caused by alkaline reaction of soils and increased content of carbonate in the fine earth. That

is why, different-aged soils of mining heaps of the quarry investigated are composed of carbonate containing substrata, presented by thin profiles of Rhendzic Leptosols, but not by Embryonic Podzols, typical for heaps, composed of acid sands, dominated on the Russian North-West.

2. The dynamics of the soil microbiome are in full agreement with soil key properties data, accumulation of the soil carbon and nitrogen, and plant community development. The number of bacteria increased by about two orders of magnitude during this time.
3. Surprisingly, the initial set (2 years) of OTUs in the entire period of soil formation remains unchanged, accumulating more than 98% of the total microbiome in each time point. This can be explained only by assuming that at the initial stage of the soil formation the initial mineral material is inoculated with the whole microbiome set transferred from the adjacent benchmark soil sites probably with aerosols. Due to that, observed soil microbiome dynamics cannot be used as a model for the primary soil formation in the past when the “benchmark inoculum” was unavailable. On the other hand, that suggests that mineral material inoculation with benchmark soil microbiome can direct the soil formation in the required direction and could be used in the real practice of the soil restoration. Moreover, it is necessary to take into account that the landscape of Izhora upland is intrazonal – Rhendzic Leptosol, so the microbiome of benchmark soil could be familiar with very initial soils due to pedoenvironmental specificity.
4. Despite the initial OTUs set conservation during the whole chronoserries, analysis of beta-diversity (weighted and unweighted) demonstrated a clear differentiation of microbiomes of all time points. This suggests that underlying taxonomic structure variations can be attributed to variation of the minor taxa set composition (explains unweighted beta-diversity) and variation in abundances of major taxa (explains weighted beta-diversity).
5. Analysis of the statistically significant changes in taxa abundances shows that the most significant differences occur in *Proteobacteria*, *Chloroflexi*, *Acidobacteria*, and *Actinobacteria*, but majority of these phyla have different sets of OTUs demonstrating opposite trends (increasing and decreasing during the soil formation).
6. The microbiome of carbonate soils in all cases of benchmark and newly formed soils is familiar because of the uniform zonal environmental conditions in geochemically specific landscape of Izhora upland. It should be taken into account that specialists will elaborate new

technologies of soil reclamation in case of humid environment chronologically based on carbonate-enriched heap mining materials.

Funding: This study was supported by Russian Foundation for Basic Research 17-16-01030 (field research, soil diagnostics), Russian Foundation for Basic Research 19-416-890002 (soil laboratory analyses), and by the state assignment of ARRIAM (Theme No. 0664-2019-0016) (sequencing and data analysis).

Conflict of interest: Authors declare no conflict of interest.

Author contributions: E. A.: investigation, conceptualization; A. Z.: investigation; A. K.: investigation, methodology; A. Ks.: investigation, writing—original draft; E. Av.: writing, project administration.

Data availability statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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