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The Prokaryotic Complex of Modern and Buried Soils on the Kamchatka Peninsula

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Abstract: A prokaryotic heterotrophic mesophilic community was studied in volcanic soil samples from Kamchatka. A phylogenetic and physiological characterization of the prokaryotic complex of modern and buried soils of the Kamchatka Peninsula is given. Volcanic Paleolithic soils (2500 and 11,300 years old) and their modern equivalents were investigated. It was found that the biomass of metabolically active prokaryotes in modern volcanic and Paleolithic soils reached 50 and 40 μg/g, respectively. The proportion of archaea in the metabolically active prokaryotic complex varied from 20% to 30% and increased in variants with the application of the nitrogen-containing biopolymer chitin. The application of the additional resource to paleovolcanic soils led to an incremental increase in the proportion of metabolically active prokaryotes, which reached 50% of the total prokaryotic biomass detected, indicating the high metabolic potential of the considered soils. Phylogenetic structure characteristics of the prokaryotic metabolically active component of modern and buried volcanic soil were established by molecular biology methods (metagenomic analysis, FISH method). The phylum *Proteobacteria* (74%), *Acidobacteria*, and *Actinobacteria* (14% combined) were dominant in modern soils; phylum *Acidobacteria* (51.8%) was dominant in paleosoils, whereas *Chloroflexi* (21%) and *Proteobacteria* (9%) were subdominant. It was determined that the potential activity of the microbial hydrolytic community, as measured by the relative response to the added resource (chitin), was found to increase in a series from modern to paleovolcanic soil. It was demonstrated that several key genes of the nitrogen cycle responsible for the processes of molecular nitrogen fixation, nitrification, and denitrification (nifH, amoA, nirK) were present in both modern and buried horizons.

Keywords: volcanic soils; metabolically active prokaryotic complex; fluorescence in situ hybridization (FISH); high-throughput sequencing

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1. Introduction

In recent years, microbiological research on the Kamchatka Peninsula has focused on extreme habitats and the presence of extremophile microorganisms [1–4]. Extreme habitats and the prokaryotic communities that inhabit them are of greatest interest to researchers. There are numerous publications on the study and identification of thermophilic, acidophilic, or alkaliphilic microorganisms from geothermally active areas of Kamchatka [1,2,5–8]. Microbiological studies of associated thermal habitats such as fumaroles, hot springs, geysers, and solfataras [9] have shown that thermal habitat communities can exist autonomously and do not depend on the external environment [9]. Conditions for the preservation of microorganisms from thermal springs and fumaroles in

the surrounding permafrost form in those regions where active volcanism and geothermal activity occur in the zone of permafrost spreading [10]. Some researchers have noted a greater productivity for soils formed on volcanic sediments in the tropical zone [11]. The greatest number of prokaryotic rRNA genes in such soils is concentrated in the upper, most organic-rich horizons. Volcanic soils in the tropics are mainly dominated by Proteobacteria and *Acidobacteria* phyla (groups *Acidobacteriales*, *Solibacterales*). Less attention is paid to soils and microorganisms inhabiting them far from active volcanic centers. Nevertheless, the soil cover of Kamchatka as a whole is a unique natural phenomenon [12]. Modern volcanic activity strongly influences the nature of the peninsula, and, because of the interaction of volcanism with other factors of soil formation, special habitats for soil microorganisms are formed. This paper focuses on the study of the microbiological component of several soils of different ages with varying distances from active volcanic centers in the Central Kamchatka Peninsula, partially removed from the general cycle of matter. Such buried soils, in our opinion, might be considered as natural depositories capable of preserving microorganisms with great biotechnological potential.

The aim of this work was to phylogenetically and physiologically characterize the prokaryotic complex of modern and buried soils of the Kamchatka Peninsula.

2. Materials and Methods

The studies were conducted in the northern part of the Kamchatka Peninsula (Figure 1a). Two sections were plotted: at the foot of volcano Shiveluch (Figure 1b), one of the largest and most active volcanoes of the peninsula [13], and at approximately 100 km from active volcanoes, in the middle ridge of Kamchatka on the southwestern foot of the extinct volcanoes Alnei–Chashakonda massif (Figure 1c). In both cases, the soil profile is a fractional interstratification of buried soil horizons and volcanic ash and is identified as a soil-pyroclastic cover (SPC).

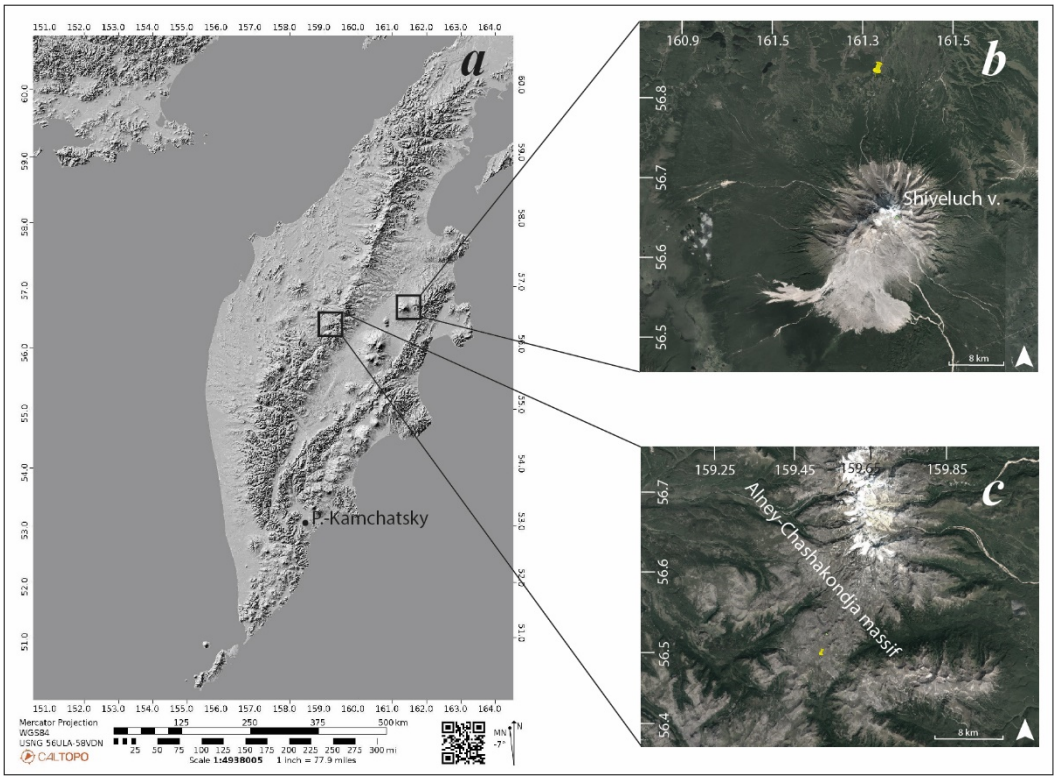

Figure 1. Geographic location of the soil sections: (**a**)—map of the Kamchatka Peninsula; (**b**)—Shiveluch volcano area; (**c**)—Alney–Chashakonja massif. Caltopo DEM topographic base (**a**), Google Earth (**b**,**c**). The position of the sections under study is shown with yellow marks.

The first section is located at the northern foot of the Shiveluch volcano, 20 km from its peak (56°49′42″ N 161°19′28″ E). The SPC is formed on deposits of a large clastic avalanche dated 11.3 ka BP in a stone-birch forest [14]. Figure 2a,b demonstrates clearly that, near the active volcano, where the maximum frequency of ashfalls is observed, the soil profile is saturated with volcanic material of different dimensions, from fine sand to gravel and lapilli pumice. A large amount of pyroclastic material also leads to an increase in the thickness of the entire section, with the soil interlayers occupying a subordinate position. Two horizons (modern and buried) of Aluandic Andosols Dystric stratified ochre volcanic soil (IUSS Working Group, 2015) were sampled to characterize the prokaryotic complex. The characteristics of the samples (Shiveluch) are presented in Table 1.

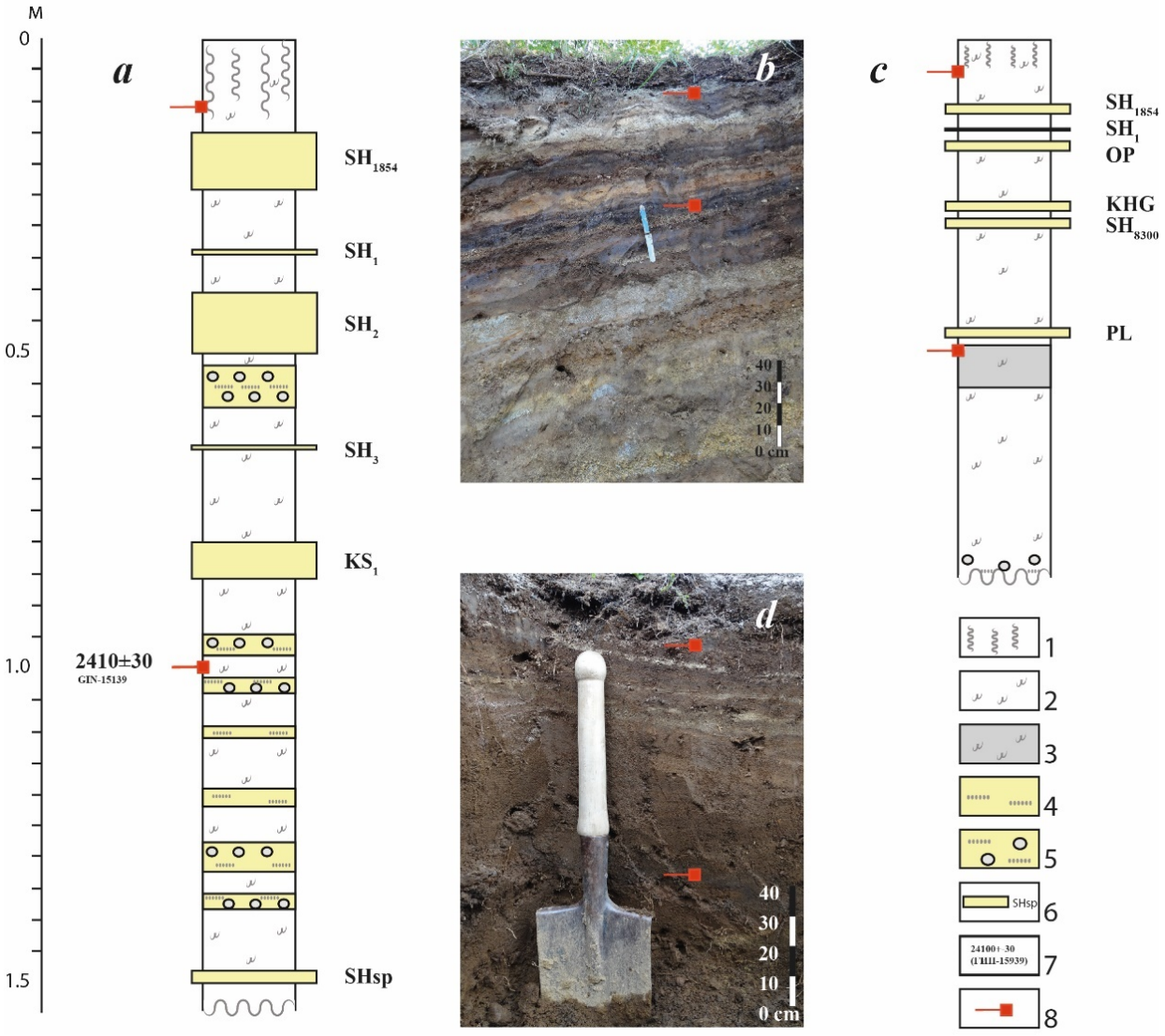

Figure 2. Structure of the studied soil profiles: (**a**,**b**)—volcanic stratified ochre soil (Shiveluch), (**c**,**d**)—volcanic humus-ochre soil (Tigil). 1—sod; 2—soil horizon; 3—highly humus buried soil horizon; 4–6—volcanic ashes: 4—volcanic sand, 5—gravel and lapilli of volcanic pumice, 6—marking indices of ash interlayers according to [13,15]; 7—radiocarbon age and laboratory sample number; 8—sampling sites. Ashes from volcanoes: Shiveluch (SH), Mutton Amphitheater on Opala Volcano (OP), Hangar (KHG), Xudach (KS), Ploskaya (PL). Photo by M.M. Pevzner (**b**), T.D. Karimov (**d**).

Table 1. Characteristics of the tested samples.

Name of Soil	Explored Horizon	Sampling Depth, cm	C_{total}	pH_{H2O}	Age (Yrs.)
Volcanic stratified ochre	Modern	10–15	21.0	5.8	modern
	Buried	110	25.5	6.2	2500
Volcanic humus-ochre	Modern	5–10	19	5.5	modern
	Buried	35–40	14	5.7	11,300

The second section was plotted in the valley of the Tigil River, whose headwaters are located in the massif of extinct volcanoes of Alnay–Chashakonja (56°29′52″ N 159°31′03″ E). The SPC is formed on lava deposits of Quaternary period in the mountain tundra zone. Figure 2c,d shows that the amount of volcanic ash in the section decreases considerably at a significant distance from active volcanoes. They noticeably decrease in thickness and are represented only by thin sands. The total thickness of the soil profile also decreases. At the same time, the thickness of organogenic soil horizons in the section increases considerably. The soil is characterized as poorly developed volcanic humus-ochric, Aluandic Andosols Dystric [16]. Two soil samples ("Tigil") were collected from the modern and buried horizons (Table 1).

Description of the section of volcanic stratified ochre soil (Shiveluch).

A depth of 15 cm—turf and loose semi-decomposed forest litter; 10 cm—dirty-white, loose, coarse-grained sand with a single gravel (SH1854); 17 cm—dark brown soil horizon, loamy, almost structureless with light layers of unidentified ash; 12 cm—the horizon of marking volcanic ash, stratified, in the upper part light fawn fine-grained to siltstone, in the lower part dark fawn of coarser granulation (SH2); 25 cm—the soil horizon is brownish-gray, with a large number of organic residues of varying degrees of decomposition, lumpy structure, loose, intertwined with a dense network of roots. There is a marking layer of ash of light fawn color, fine-grained (SH3); up to 6 cm—marking layer of ash, ochreous siltstone (KS1); 60 cm—interlayer of ash horizons and buried soil (grayish-ochre and grayish-brown) lightly loamy, desalinated; 1–1.5 cm—bright bluish-gray fine-grained sand, hardened along the lower border.

Description of the section of volcanic humus-ochre soil (Tigil).

A depth of 3 cm—turf, 1 cm from the roof light gray siltstone with an admixture of fine-grained sand (unidentified ash); 9 cm—humus horizon, dark gray to brown, slightly structured, powdery, loose with stratified layers of black fine-grained sands of basalt composition; 0.2–1.2 cm—pinkish-light gray siltstone with an admixture of fine-grained sand (SH1); 2 cm—humus horizon, dark gray to brown, slightly structured, powdery, loose, with stones not rolled in diameter up to 0.5 cm; 0.5 cm—bright creamy white siltstone with biotite (OP); 6 cm—dark coffee, with an unclear structure, with stones and volcanic sand of different composition; 1–2 cm—fawn siltstone (KHG); 2 cm—dark coffee, with an unclear structure; up to 2 cm—pale gray fine-grained sand of the "salt and pepper" type with rusty fine grains (SH8300); 54 cm—grayish-ochreous, loamy, fragile lumpy–powdery structure. Decayed, with fragments up to 15 cm in diameter throughout the power. At a depth of approximately 50 cm, a dark coffee to black high-humus horizon.

Radiocarbon dating of buried soils was carried out at the Geological Institute of the Russian Academy of Sciences (Moscow) according to the standard soil methodology, which is discussed in detail in Pevzner, 2015 [17]. Measurements were carried out on radiometric installations with a counting efficiency of approximately 50% in non-replaceable calibrated cuvettes with working volumes from 1.5 to 22.03 mL. E-5 and background samples were used as standards. The measurement of the age of one counting drug was carried out at least twice (one measurement lasts approximately a day), with an interval between them of 5–10 days on average. With a satisfactory score (the values of different measurements converge within the error), the average radiocarbon age of the sample was taken. The radiocarbon dates were calibrated to calendar age using Oxford University calibration

software OxCalv4.4.2IntCal 20 [18,19]. The studied soil horizon of the volcanic stratified ochre soil at the foot of Shiveluch volcano was formed approximately 2.5 thousand years ago (calBP). The age of the volcanic hummocky-ochre soil in the Tigil River valley was estimated based on the stratigraphy of marker ashes. The age of the Sopok Plosky eruption, resulting in the deposition of marker ash PL, is estimated at 11,650 years calBP [15]. Since the soil is deposited directly beneath the PL ash (Figure 2c), its age is approximately 12 thousand years calBP. Soil microbial succession was initiated by humidification (soil water pressure was −3.2 kPa) and application of 0.6% biopolymer chitin suspension (0.3 g/5 g soil), as well as crude oil in an amount largely exceeding the MAC of 10% (1 mL/5 g soil). The soil weight was 5 g. Purified chitin was obtained from Sigma-Aldrich. Oil was obtained from the Azov–Kuban oil-and-gas-bearing basin (OGB, Kubanskaya area field, Well C-1, Tables 2 and 3).

Table 2. Physico-chemical characteristics of the oil in this study.

Indicator Name	Value
Sampling depth, m	2800–2832
Kinematic viscosity at 200 C, mm^2/s	1.5 (4.9)
Flash point, 0 C	61
Mass fraction of sulfur, %	0.5
Water content	traces
The contents of mechanical impurities, %	None
Water-soluble acids and alkali content	None
Density at 150 C, g/cm^3	0.835
Iodine content	0.4
Cetane number	51
Color	brown

Table 3. Chemical composition of the oil used in this study.

Compound class	Compound	Formula	Molecular Weight
Paraffins	n-Hexanes	C6H14	87.186
	2-methylpentane	C6H14	87.186
	n-heptane	C7H16	100.205
Naphthenes	Cyclohexane	C6H12	84.162
	Methylcyclohexane	C7H14	98.189
Aromatic compounds	Benzene	C6H6	78.114
	Toluene	C7H8	92.141

The use of two substrates (oil and chitin) is methodologically justified by different carbon-to-nitrogen ratios (C:N). This should lead to different degrees and speeds of substrate utilization and allows for assessment of the metabolic potential of the soil microbial community.

The following parameters were used as indicators of microbial decomposition of chitin and oil in soils: dynamics of bacterial numbers and biomass, assessment of metabolically active prokaryotic cells, presence or absence of functional genes characterizing the main metabolic reactions associated with N and C cycles in the system.

Epifluorescence microscopy using different fluorochromes—acridine orange, Sy3—was used to determine the total number and the number of metabolically active cells in the studied samples [20]. The total number of prokaryotes was determined using acridine orange dye, which reacts with cell DNA. Soil samples were pre-treated with Bandel in Sonopuls

HD 2070 (Germany) for 2 min and 50% power for better cell desorption. Assessment of diversity and abundance of the metabolically active representatives of individual phylogenetic groups of microorganisms in the soils was carried out by in situ hybridization with rRNA-specific fluorescent-labeled oligonucleotide probes (fluorescent in situ hybridization, FISH) [21–24]. In this study, we used an array of probes specific for representatives of Archaea and Bacteria domains, as well as representatives of several phylogenetic groups (*Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *α-Proteobacteria*, *β-Proteobacteria*, *γ-Proteobacteria*, *δ-Proteobacteria*, *Planctomyces*, *Verrucomicrobia*, *Euryarchaeota*, *Crenoarchaeota*, and *Thaumarchaeota*). This allowed for analysis of the bacterial and archaeal community of the soils under study. Hybridization temperature regime and hybridization and washing buffers were optimized for each bacterial phylogenetic group [25]. Prepared samples were observed using PrimoStar microscope (Zeiss, Germany) with a light filter (AmScope, Irvine, CA, USA). Prokaryotic biomass (B) was calculated according to Formula (1):

$$B = N \times 2 \times 10^{-14}, \quad (1)$$

where N is the number of prokaryotic cells in 1 g of soil sample of bacteria in 1 g of sample, and the biomass of dry matter for 1 bacterial cell with volume of 0.1 μm^3 is 2 × 10–14 g [20]. The specific gravity of microorganisms was taken as 1 g/cm^3; the water content of the cells was 80%.

Total DNA extraction followed by sequencing was performed for all soil sample types (buried and modern). Metagenomic DNA was extracted from a 0.5 g soil sample using a specialized DNeasy Power Lyzer Power Soil Kit (MoBioLaboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's protocol. The nucleotide sequences of V3-V4 region of variable sections of 16S rRNA gene from metagenomic DNA samples were determined by high-throughput sequencing [26]. Sequencing was performed on Illumina Miseq sequencer by Evrogen Research Company (Moscow, Russia) with 39 h read time and 8 mln pair-end reads. Then, a file with forward and reverse reads from both ends of DNA (301 cycles from each end of the fragments) was generated, which was a textual description of the primary structure of linear macromolecules in the form of a monomer sequence. Sequencing data processing (FASTQ files) was performed using the DADA2 package functions for the programming language R.

The software tools were used to perform the following: check sequencing quality and create a library of sequences; generate OTUs (de novo OTU picking) with 97% similarity threshold; remove singletons ("singletons" are OTUs containing only one sequence) and sequences belonging to plant chloroplasts; remove chimeras.

In addition, random samples were created from each table: 2500 from initial coverage, 100,000 from final coverage, 2500 step, with the number of replicates—5. For each sample, Sørensen–Dyce and Bray–Curtis pairwise distance matrices were calculated. We calculated Shannon, Simpson, Pielu, and Chao alpha diversity scores using functions from the scikit-bio package for the Python programming language for each sample. Values for the same depth grouped by indicator and sample were averaged.

Sequences were visualized using Krona [27].

The abundance of ribosomal genes in bacteria and archaea was estimated by real-time polymerase chain reaction (RT-PCR) [28]. The reaction was performed in a DTLite4 DNA-Technology amplifier. The reaction mixture was prepared from Super Mix Eva Green (Carlsbad, CA, USA). The instrument was calibrated using the dependence of fluorescence intensity on the logarithm of DNA concentrations of standard solutions. Solutions of cloned fragments of ribosomal operon from *Escherichia coli* strain K12 were used as standards for 16S rRNA gene concentration for bacteria, and those of *Halobacterium salinarum* strain FG-07 were used for archaea. For each experimental variant (sample), the reactions were performed in three replicates and with negative control (sample without DNA matrix). Data processing was performed using the Real time_PCR software package. All DNA-specific primers used in the work, as well as reaction protocols (amplification temperature profile), are described in Table 4.

Table 4. qPCR primers and temperature conditions used to determine the presence of genes in the studied samples.

Primer	Sequence (5′–3′)	Temperature Profile
Eub: Eub338f Eub518r	ACTCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	95 °C 3 min; 95 °C 10 s, 50 °C 10 s, 72 °C 20 s—49 cycles; 72°C 20 s
Arch: arc915f arc1059r	AGGAATTGGCGGGGGAGCAC GCCATGCACCWCCTCT	95 °C 3 min; 95 °C 10 s, 50 °C 10 s, 72 °C 20 s—49 cycles; 72 °C 20 s
NifH: Forward Reverse	GGTTGTGACCCGAAAGCTGA GCGTACATGGCCATCATCTC	94 °C 1 min; 94 °C 30 s, 50 °C 1 min, 72 °C 30 s—40 cycles; 72 °C 10 min
amoA(AOB): amoA-1F amoA-2R	GGG GTT TCT ACT GGT GGT CCC CTC KGS AAA GCC TTC TTC	95°C 10 min; 94°C 45 s, 58°C 45 s, 72°C 45 s—39 cycles; 95 °C 15 s, 60 °C 30 s, 95 °C 15 s
amoA(AOA): 23F CrenamoA616	ATG GTC TGG CTW AGA CG GCC ATC CAB CKR TAN GTC CA	94°C 45 s, 55°C 45 s, 72°C 45 s—39 cycles; 95 °C 15 s, 60 °C 30 s, 95 °C 15 s
nirK: nirK876 nirK1040	ATY GGC GGV CAY GGC GA GCC TCG ATC AGR TTR TGG TT	95°C 15 min; 95°C 15 s, 60°C 30 s, 72°C 30 s, 80°C 15 s—40 cycles; 95 °C 15 s, 72 °C 30 s
AlkB: alkB-1f alkB-1r	AAYACNGCNCAYGARCTNGGNCAYAA GCRTGRTGRTCNGARTGNCGYTG	94 °C 3 min, 94 °C 1 min, 61 °C 1 min, 68 °C 45 s—26 cycles; 68 °C 10 min

To better characterize the nitrogen cycle in the studied microbial communities, the genes responsible for the nitrification (ammonium oxidation) process were also amplified: *amoA* (AOB) for bacteria [29] and *amoA* (AOA) for archaea [30]. The reactions used a series of dilutions of a known number of copies of linearized plasmid (PCR4-TOPO, Aberdeen, Scotland) containing the *amoA* gene *Nitrosospira multiformis* ATCC25196. The following reaction protocol was used to amplify ammonium oxidizing bacteria: 95 °C 10 min; 94 °C 45 s, 58 °C 45 s, 72 °C 45 s—39 cycles; 95 °C 15 s, 60° C 30 s, 95 °C 15 s. The protocol for amplification of ammonium oxidizing archaea was: 94 °C 45 s, 55 °C 45 s, 72 °C 45 s—39 cycles; 95 °C 15 s, 60 °C 30 s, 95 °C 15 s.

Amplification of the genes responsible for the denitrification process of *nirK* [31] (Table 4) was carried out according to the following scheme: 95 °C 15 min; 95 °C 15 s, 60 °C 30 s, 72 °C 30 s, 80 °C 15 s—40 cycles; 95 °C 15 s, 72 °C 30 s. The *nifH* gene was amplified to determine the presence of the gene responsible for the ability to supply available nitrogen to the community (nitrogenase activity) [32]; the amplification temperature profile is shown in Table 4. DNA solutions extracted from cultures of *Bacillus cereus* and *Azotobacter chroococcum* were used as gene concentration standards for *nirK* gene and *nifH* gene copies, respectively.

The primer system described in Smith et al., 2013 [33] was used to identify functional genes indicative of n-alkane degradation processes. *Streptomyces violaceus* (*sec. Roseus: ser. Roseoviolaceus*) culture capable of using hydrocarbons as a single food source (toluene) was used as a standard for detection of *alkB* gene copies. The following protocol for the amplification was used: 1 cycle: 94.0 °C—5 min; 30 cycles: 94.0 °C—1 min; 60 °C—1 min; 72 °C—1 min; 1 cycle: 72 °C—3 min; 1 cycle—4 °C—cooling [34] (Table 4).

3. Results

The biomass of prokaryotic communities in the modern and buried horizons of the studied soils was determined by the direct accounting method. A greater prokaryotic biomass in the modern horizons than in the buried ones was observed for the studied samples (Figure 3). The addition of chitin leads to an increase in biomass in both buried and

modern soil samples. However, the relative response of the microbial community of the buried soils to substrate application is significantly higher than that of modern soils. Similar patterns were noted by the authors earlier for the microbial hydrolytic community of buried paleochannels compared to modern chestnut soils [20]. When polysaccharide was applied to chestnut paleosoils, the proportion of metabolically active prokaryotic cells reached 50% of the total detectable prokaryotic community, and the response to the substrate application was more intense compared to modern soils. Similar results were obtained for the structure of the prokaryotic complex of Antarctic permafrost [35].

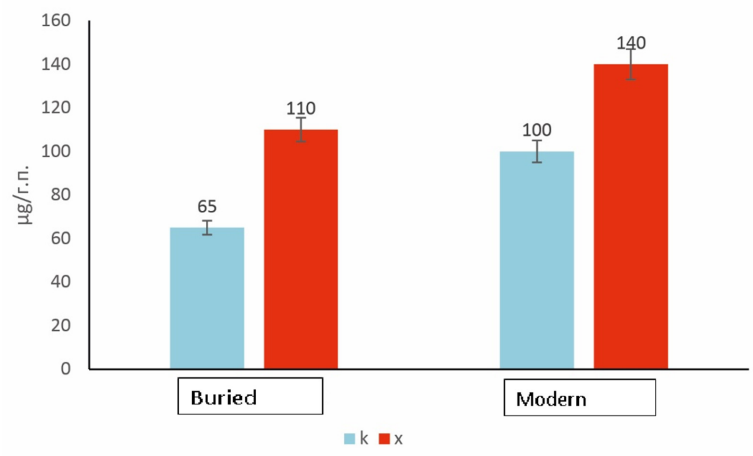

Figure 3. Total biomass of prokaryotes in the studied samples of volcanic stratified ochre soil on the 10th day of succession initiated by addition of substrate and without it: k—control; x—chitin.

By the tenth day of the succession, the biomass of metabolically active representatives of the prokaryotic component of the sample collected from the buried horizon of volcanic stratified ochre soil moistened with water was 40 µg/g.p. (of which, 35 µg/g.p. was the bacterial component). An increase in biomass was observed in the microcosm following the addition of chitin compared to the control sample; the biomass value was 60 µg/g.p. (of which, 44 µg/g.p. was the bacterial component) (Figure 4).

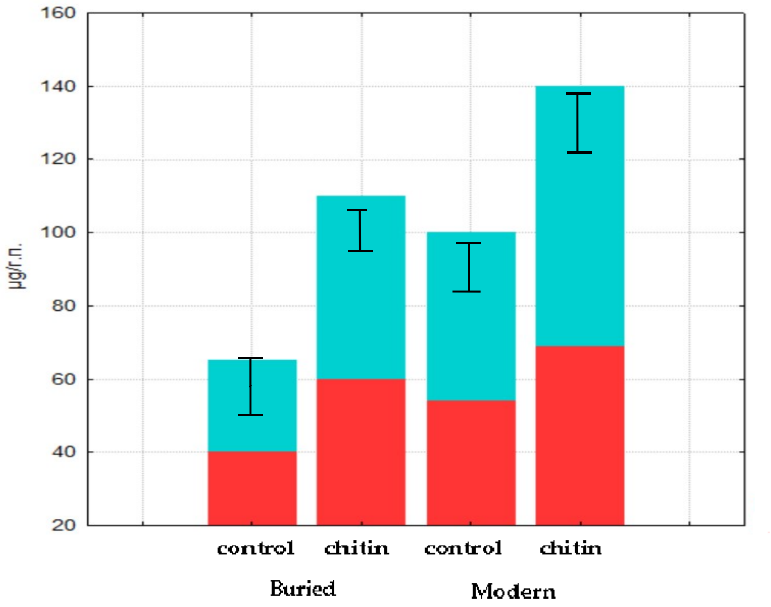

Figure 4. Biomass of metabolically active prokaryotes from the total biomass in the studied soils on the 10th day of the succession, initiated by adding substrate and without it: blue—cells unhybridized by probes; red—metabolic activity cells.

By the thirtieth day of the succession, the metabolically active biomass of the sample of the buried horizon of volcanic stratified ochre soil moistened with water was 57 μg/g.p. (of which, 48 μg/g.p. was the bacterial component). The prokaryotic community biomass was 32 μg/g.p. (of which, 29 μg/g.p. was the bacterial component) in the variant with chitin by the thirtieth day of the succession. Thus, our results confirm the data obtained earlier for samples of chestnut modern and paleo-soil soils. The proportion of metabolically active prokaryotic components in the buried soil horizons reached 50% of all of the detected biomass when the microbial community was reactivated by the resource.

The number of copies of bacterial rRNA genes in the studied soils was determined using a real-time polymerase chain reaction. The gene copy number in the soils was measured dynamically during the succession. The variants with oil and chitin addition, as well as the control, were considered. The values of bacterial genes in the buried horizon were comparable to those of modern soils, reaching n × 10^{13} genes (Figure 5). The greatest response to the application of the resource was noted in the variants with chitin in the buried sample. This coincides with the results of total and metabolically active prokaryotic biomass in the buried soil samples with biopolymers, indicating a possible high metabolic potential of the studied soils.

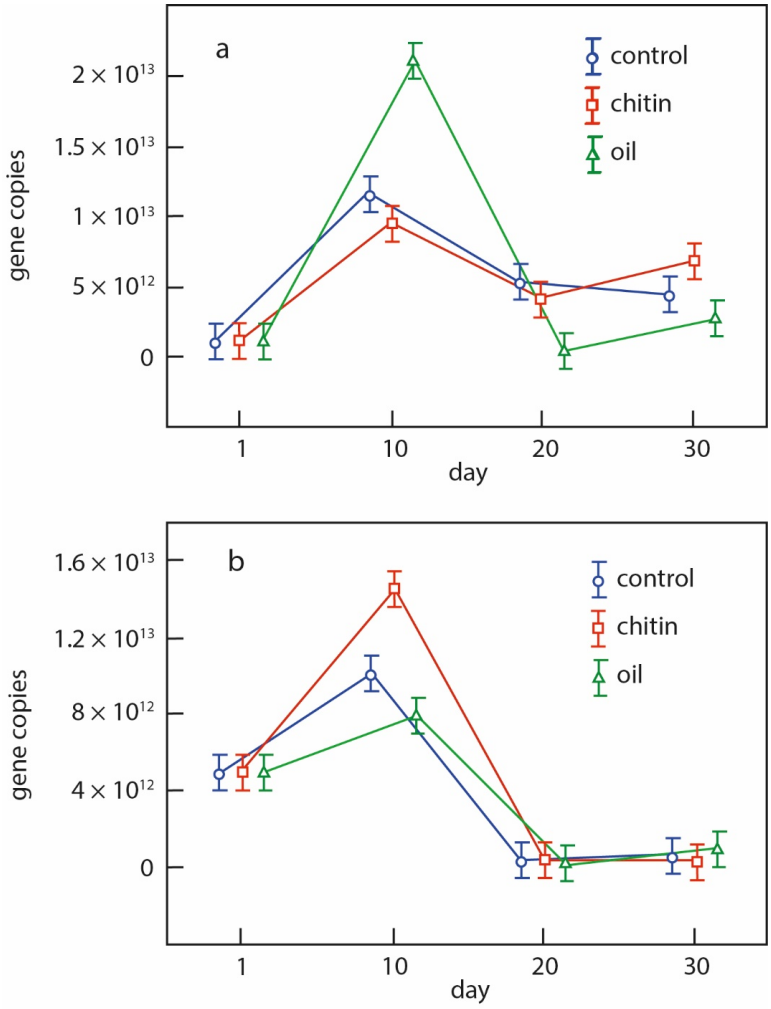

Figure 5. Dynamics of 16S RNA bacterial gene copy number with and without substrate addition: k—control; x—chitin; n—oil in different horizons of volcanic stratified ochre soil: (**a**) in the modern horizon (LS means. Current effect: F(6, 12) = 48.215; p = 0.00000. Decomposition of III types. Vertical bars denote 0.95 confidence intervals); (**b**) in the buried horizon (LS means. Current effect: F(6, 12) = 15.926; p = 0.00004. Decomposition of III types. Vertical bars denote 0.95 confidence intervals).

The phylogenetic structure of the prokaryotic communities of two studied soils—volcanic stratified ochre and volcanic humus-ochre—was determined by the FISH method and metagenomic analysis. Significant differences in the structure of the bacterial complex of modern and buried soils were revealed.

The microbial community structure of the studied samples was determined using Krona software (Figure 6). For the modern horizon, it was determined that phylum *Proteobacteria* was dominant (74%); representatives of phyla *Actinobacteria* and *Acidobacteria* (14% together) were included in the moderate abundance groups, and the minor group included *Gemmatimonadetes*, *Chloroflexi*, and *Verrucomicrobia* (8% together) in this community (Figure 6B). Representatives of the other domain groups are only marginally represented, totaling less than 1%.

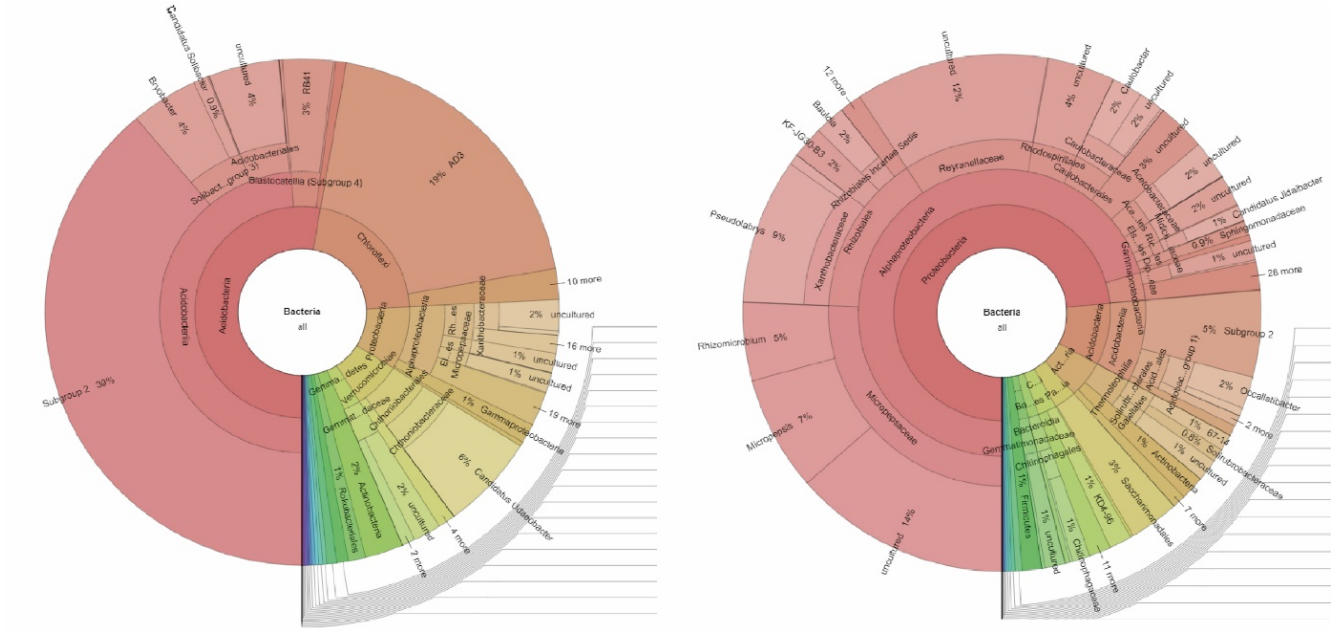

Figure 6. Phylogenetic map of the structure of the bacterial component (%) of the buried (**A**) and modern (**B**) horizons of volcanic stratified ochre soil.

The biomass of metabolically active *Proteobacteria* phylum was 17 μg/g of the sample. Among *Proteobacteria*, representatives of class *Alfaproteobacteria* and orders (families) *Ellin329*, *Rhodospirillales* (*Rhodospirillaceae*, *Acetobacteraceae*), *Rhizobiales* (*Hyphomicrobiaceae*, *Bradyrhizobiaceae*), *Caulobacterales* (*Caulobacteraceae*), *Rhodospirillales* (*Acetobacteraceae*), and *Sphingomonadales* (*Erythrobacteraceae*) dominated. The classes *Betaproteobacteria* and *Gammaproteobacteria* are less represented.

Saccharibacteria (candidate phylum *TM7*) were detected in significant numbers.

For the *Acidobacteria division* (biomass of metabolically active representatives was 10 μg/g of the sample), the most characteristic orders were *Acidobacteriales* (*Acidobacteriaceae*, *Koribacteraceae*) and *Solibacterales*.

The *Actinobacteria* group having a biomass of metabolically active representatives in the amount of 8 μg/g of the sample was mainly represented by the class *Thermoleophilia* and its orders (*Solirubrobacterales*—family *Patulibacteraceae*, *Gaiellales*—family *Gaiellaceae*). The *Acidimicrobiales* (*EB1017*) and *Actinomycetales* (*Nocardioidaceae*) orders were also identified among *Actinobacteria* representatives.

Among the *Chloroflexi*, the classes *Ellin6529* and *Anaerolineae* (*GCA004*) were identified.

Members of the family *Chitinophagaceae* of the order *Bacteroidetes* were detected in significant numbers.

Clostridiales (*Clostridiaceae*) was the most common among *Firmicutes*, and *N1423WL* among *Gemmatimonadetes*. The phylum *Nitrospirae* was represented by the class *Nitrospira* (order *Nitrospirales*, family *Nitrospiraceae*).

The archaeal community complex of the modern volcanic stratified ochre soil horizon was dominated by *Crenarchaeota* (*NRJP*) whereas *Cenarchaeales* was only minor. Among the *Euryarchaeota*, representatives of the class *Thermoplasmata* (order E2, family *Methanomassilii coccaceae*) were identified.

For the buried horizon (Figure 6A), the phylum *Acidobacteria* was dominant (51.8%), with subdominants being *Chloroflexi* (21%), *Proteobacteria* (9%), and *Verrucomicrobia* (7%). Representatives of *Actinobacteria* and *Gemmatimonadetes* phyla held minor positions in the structure of the studied community (4% together). The conditions for the existence of microorganisms change greatly during the transition from modern soils to their buried analogues. First of all, this affects the content of the resource available to microbes. Paleosols are characterized by a lower content of organic matter, which entails a change in the structure of the microbial community of soils. Thus, earlier, the authors found that, in the microbial complex of buried chestnut soils, in comparison with their modern counterparts, the number of spore-forming actinobacteria increases noticeably. In addition, with the introduction of chitin, this pattern was significantly enhanced [20].

The biomass of metabolically active *Acidobacteria* representatives in paleochannels reached 20 μg/g of the sample. *Acidobacteria* were represented by classes *DA052* (order *Ellin6513*), *Chloracidobacteria*, *Chloracidobacteria* (*o__RB41*), orders *Acidobacteriales* (*Acidobacteriaceae*, *Koribacteraceae*), and *Solibacteres* (*Solibacterales*).

The following representatives were found in large numbers: *Dormibacteraeota* (formerly known as *AD3*) from the phylum *Chloroflexi* typical for deep soil horizons with a reduced organic carbon content; also active hydrolytes from division *Verrucomicrobia* (class *Spartobacteria*, order *Chthoniobacterales*, family *Chthoniobacteraceae*); and, finally, class *Methylacidiphilae* (*S-BQ2-57*) from *Verrucomicrobia.*

Proteobacteria with a biomass of metabolically active representatives in the amount of 8 μg/g of the sample, mainly *Alphaproteobacteria* (order *Ellin329*), as well as orders (*Rhodospirillales*, family *Rhodospirillaceae*) and *Rhizobiales* (*Hyphomicrobiaceae*), were detected. Among *Betaproteobacteria*, the order *Ellin 6067* was detected.

Actinobacteria were represented by the classes *Acidimicrobia* (*Acidimicrobiales*, family *EB1017*) and *Thermoleophilia* (order *Gaiellales*, family *Gaiellaceae*).

Gemmatimonadetes (*Gemmatimonadetes*, order *N1423WL*, family *c__Gemm-1*), *Nitrospirae* (*Nitrospira*, order *Nitrospirales*, family *0319-6A21*), and *Bacteroidetes* (*Sphingobacteriia* order *Sphingobacteriales*) were identified.

The archaeal community complex of the buried volcanic stratified ochre soil horizon was dominated by *Euryarchaeota*, mainly *Methanomicrobia* (*Methanocellale*). The representatives of *pGrfC26* were typical for *Crenarchaeota* in the buried horizon. *Thaumarchaeota* were represented by the order *Cenarchaeales* (*SAGMA-X*).

Biodiversity indices (i.e., alpha-diversity) were calculated for each sample using the Python scikit-bio software package. For the modern horizon, the following indices were calculated: the Chao1 index was 669.5, Pielu index ("alignment" of the community) = 0.6, Shannon index = 5.65, Simpson index = 0.95. For the buried horizon, the Chao index was 448.3, Pielu index = 0.59, Shannon index = 5.26, Simpson index = 0.91.

The addition of chitin to the studied samples leads to a change in the structure of the metabolically active prokaryotic component of the microbial community. The number and biomass of actinobacteria increase, whereas the number of proteobacteria decreases (Figure 7). More evidence of the ability of prokaryotes to synthesize a variety of instances of depolymerization was obtained [36,37]. A high chitinolytic activity of *Actinobacteria* is known from the literature [38]. Actinomycete complexes have a high chitinolytic activity in various soils [39,40] The intensity of prokaryotes-destroyers, as well as that of all microorganisms, strongly depends on environmental factors, including temperature, moisture, redox conditions, etc.

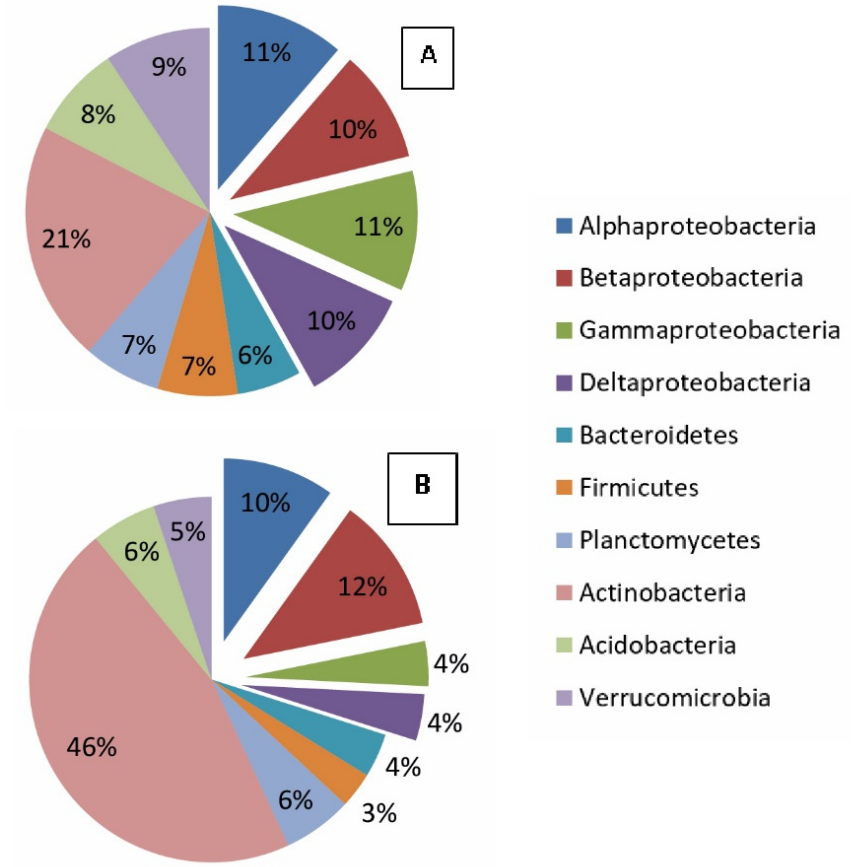

Figure 7. Structure of the metabolically active bacterial complex of the buried horizon of stratified ochre soil on the 10th day of succession with the addition of substrates: (**A**)—control; (**B**)—chitin.

In parallel, the functional activity of the prokaryotic community was assessed in the studied soil samples by detecting the presence of nitrogen cycle genes in the system: *nifH*—nitrogenase activity, *amoA* (AOB)—ammonium oxidizing bacteria monooxygenase activity, *amoA* (AOA)—ammonium oxidizing archaea monooxygenase activity, and *nirK*—nitrate reductase activity.

As expected, the highest values for the functional gene nifH in the studied soils were observed in the control variant without the addition of nitrogen-containing polymers. Moreover, the maximum values were observed for the modern horizon and reached 1.54×10^7 gene copies/g.p. by the 30th day of succession. For the buried horizon, despite an overall increase in gene concentration by the 30th day, its maximum values were observed at the beginning of succession (2.5×10^5 gene copies/g.p.). The values of the gene content in the modern horizon were two orders of magnitude higher than in the buried. For all soils, there was a tendency in the lower content of the nifH gene when introducing substrates into the soil. This feature can be explained by the presence of nitrogen forms available to microorganisms in the soil with polymers (especially chitin), which leads to an intensification of mineralization processes and a decrease in nitrogenase activity. It is important to note the presence, albeit insignificant, of the nitrogenase gene in the deeper layers of the soil, which indicates the possible potential of microbial communities living there.

An amplification of the key nitrification gene was also carried out. For volcanic soils (both modern and buried), the presence of ammonium oxidizing bacterial and archaeal genes was detected (Figures 8 and 9).

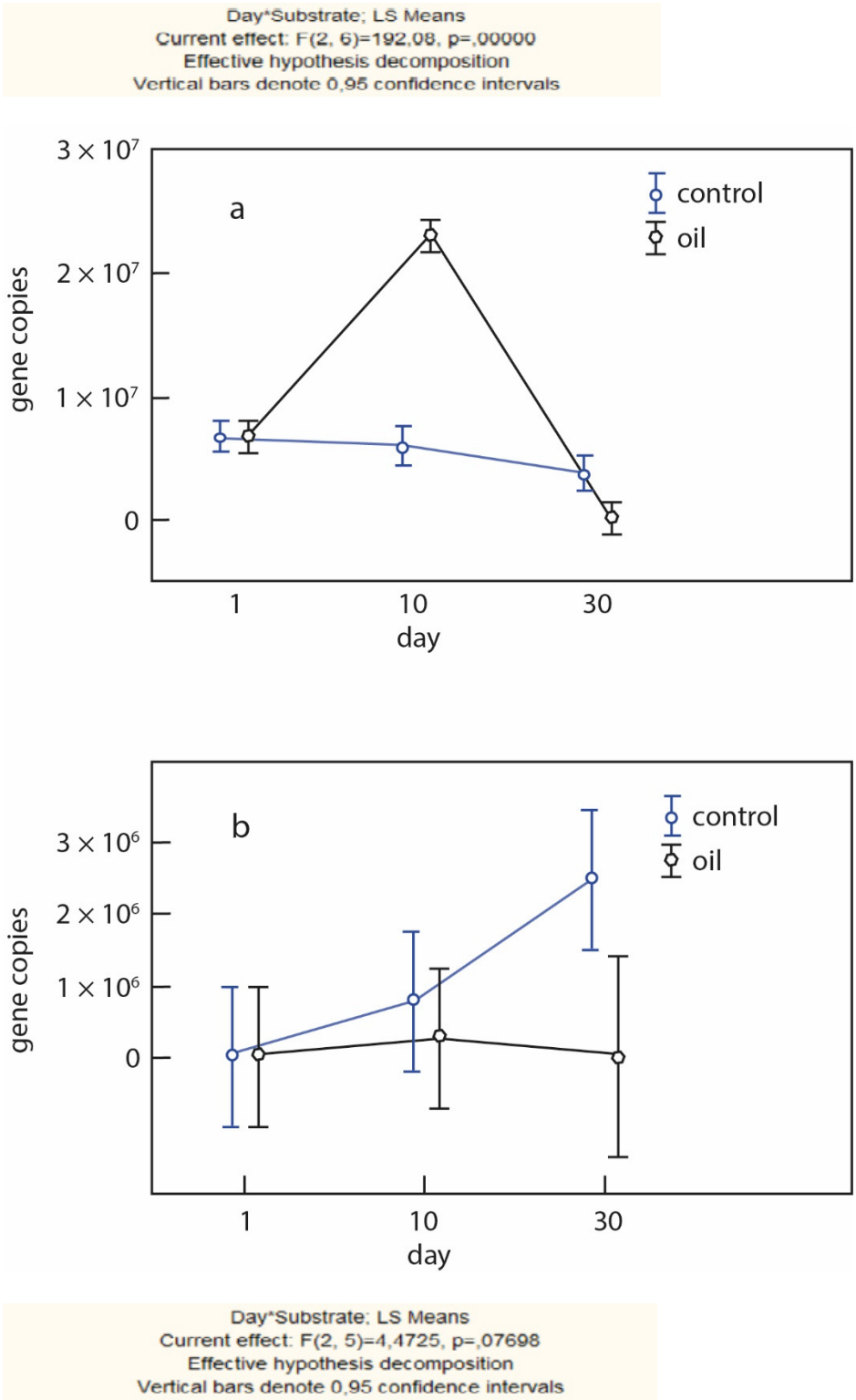

Figure 8. Dynamics of functional *amoA* genes in volcanic soils (modern horizon): (**a**)—bacteria; (**b**)—archaea.

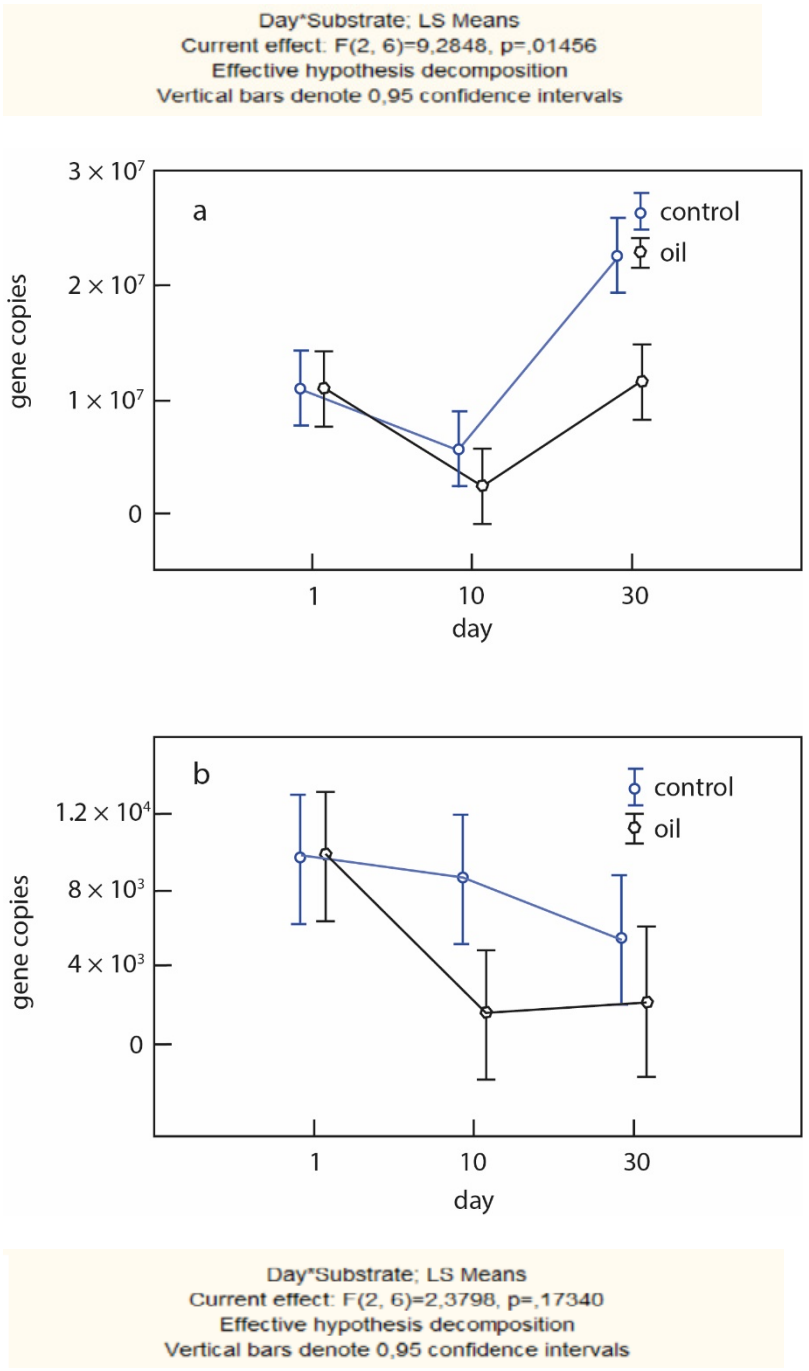

Figure 9. Dynamics of functional *amoA* genes in volcanic soils (buried horizon): (**a**)—bacteria; (**b**)—archaea.

An increase in the gene concentration in the buried samples after 30 days of succession in the control variants and the variant with oil was noted, indicating a reactivation of nitrifiers by the end of succession (Figure 9a). The dynamics of the *amoA* gene in the modern horizon of the volcanic humus-ochre soil shows its increased concentration in the bacterial complex in the variants with oil (2.3×10^7 DNA copies/g.p.) by the 10th day of succession, whereas a low content of the gene is noted in the control. In the archaeal complex, there is an increase in the gene concentration in the control variant after 30 days (2.5×10^6 DNA copies/g.p.), whereas the indicators remain very low in the variant with oil.

Based on the data obtained, it can be concluded that archaea play a leading role in the nitrification process in soils without oil (undisturbed community). The bacterial complex is capable of nitrification with a high oil contamination of the soil, and its role also increases in the lower layers of the soil profile. The contribution of archaea to the nitrification process decreases with the introduction of oil, and also decreases with depth.

Copies of the *nir K* gene, a molecular marker of denitrification, were detected in volcanic humus-ochre soil samples in all variants of the experiment (control, oil) during succession. The absolute values were the highest in the modern horizon (1.25×10^7 DNA copies/g p), whereas, in the buried horizon, they were two orders of magnitude lower (1.2×10^5 DNA copies/g p).

In all horizons, there was a reduced content of the gene in the variant with the introduction of oil. The greatest negative impact of oil was noted in the buried horizon.

The presence of *alkB*, a gene responsible for the activity of the n-alkanoxygenase enzyme, was assessed for the volcanic humus-ochre soil system. The *alkB* gene was detected in all variants; its absolute concentration was maximal in the modern horizon for a variant with oil on the 10th day of succession (2.5×10^4 DNA copies/g.p.). Thereafter, it decreased in the oil and control variants, and, after 30 days of succession, was 5×10^5 DNA copies/g.p. and 1.3×10^6 DNA copies/g.p., respectively.

There is a notable trend toward an increasing gene concentration during succession for all variants in the buried horizon. Absolute values of the gene content in buried soil by 30 days are 4.8×10^4 DNA/g.p for the control variant and 1.8×10^4 DNA copies/g.p for a variant with oil. Similar dependencies were found when considering the activity of hydrolytic (chitinolytic) microbial complexes of modern and buried soils.

4. Discussion

The work complements the previously obtained data from studies of relict microbial communities, and also reveals previously unknown features of such microbial complexes. Previously, studies of ancient communities were limited due to the imperfection of the methodological base. The applied complex of methods of classical microbiology and new molecular biological methods allows for the most complete characterization of the metabolically active prokaryotic community of buried soils. Buried soils, apparently, for the first time, became the object of a study on metagenomics. The analysis of the data of the high-performance sequencing of the 16S rRNA gene and the subsequent restoration of the functional genetic complex allowed us to identify the main dominants of the hydrolytic community of ancient soils. Presumably, the dominants of ancient hydrolytic communities may have higher activity compared to their collectible counterparts. The revealed increase in the potential activity of communities as the degree of "conservation" of the community increases opens up opportunities for the biotechnological use of strains isolated from being buried. The conducted research, apparently, is one of the most comprehensive and complete works devoted to the ancient microbial complex.

To summarize, the prokaryotic heterotrophic mesophilic community has been successfully studied in volcanic soil samples from Central Kamchatka. Paleolithic soils of different ages and their modern analogues were investigated. The age of the buried horizon of volcanic stratified ochre soil was 2500 years old and the horizon of volcanic humus-ochre soil was approximately 11,300 years old. It was found that the biomass of metabolically active prokaryotes of volcanic layered-ochre soil reached 54 μg/g in the control sample and 69 μg/g of the sample in the variant with the addition of chitin, which is approximately 40% of all detected prokaryote biomass. In paleovolcanic soil, the biomass indices of metabolically active prokaryotes were 40 μg/g and 60 μg/g, respectively. The addition of the chitin biopolymer to the paleovolcanic soil caused the indicators of the metabolically active prokaryotes share to reach 50% of the detected prokaryote biomass. Similar patterns were noted by the authors earlier for the microbial hydrolytic community of buried paleochannels compared to modern chestnut soils [20]. When polysaccharide was applied to chestnut paleosoils, the proportion of metabolically active prokaryotic cells reached 50% of

the total detectable prokaryotic community, and the response to substrate application was more intense compared to modern soils.

The proportion of archaea in the metabolically active prokaryotic complex ranged from 20% to 30% and increased in variants with resource addition, which is consistent with the results of other researchers [4].

The features and differences in the phylogenetic structure of the prokaryotic metabolically active component of modern and buried volcanic stratified ochre soil were established by molecular biological methods (metagenomic analysis, FISH method). *Proteobacteria* (74%), *Acidobacteria*, and *Actinobacteria* (14% combined) were dominant in modern soil phylum; *Acidobacteria* was dominant (51.8%), and *Chloroflexi* (21%) and *Proteobacteria* (9%) were among subdominants in buried soil phylum. Obviously, such a feature of the structure of the complex is associated with a change in the resources present in the environment and used by microorganisms. The total diversity of the studied microbiomes (alpha diversity) differs slightly in buried and modern soils. The Shannon index does not differ significantly in buried and modern soils. At the same time, the amount of OTE (species richness) in modern soils is higher than in buried ones.

The introduction of an additional substrate (chitin) into the studied soil samples caused a change in the structure of the metabolically active prokaryotic component of the microbial community. This was especially noticeable for samples of buried soils. The number and biomass of *Actinobacteria* doubled in the bacterial hydrolytic complex of stratified ochre paleosoils compared to its modern counterpart. The development of actinobacteria on such an inaccessible polysaccharide, nitrogen-containing biopolymer as chitin has been repeatedly noted by the authors [7,20]. The functional role of mycelial actinobacteria in the metabolism of chitin consists, on the one hand, of the active decomposition of this biopolymer, and, on the other hand, of the regulation of microbial hydrolytic complex activity through the production of biologically active regulatory metabolites, which occurs in a wide range of environmental parameters (moisture, temperature, organic matter, successional time).

The introduction of the substrate into both buried and modern soil reduced the content of nifH genes. This feature can be explained by the presence of nitrogen forms available to microorganisms in the soil with polymers (especially chitin), which leads to an intensification of mineralization processes and a decrease in nitrogenase activity. It is important to note the presence, albeit insignificant, of the *nifH* gene in the deeper layers of the soil, which indicates the possible potential of microbial communities living there.

An interesting feature is noted when considering the functional genes responsible for the nitrification process. For uncontaminated territories, the archaeal component of the prokaryotic community of soils plays a significant role in the process of ammonium oxidation. Similar results were obtained in the works of other authors, which provide evidence that archaea play a leading role in the oxidation of ammonium in soils [41–43]. In soils without oil, archaea play a leading role in the nitrification process. The bacterial complex is capable of nitrification with a high oil contamination of the soil, and its role also increases in the lower layers of the soil profile. The contribution of archaea to the nitrification process decreases with the introduction of oil, and also decreases with depth.

The identified patterns indicate a possible high metabolic potential of the studied soils.

5. Conclusions

The prokaryotic heterotrophic mesophilic community has been successfully studied in volcanic soil samples from Central Kamchatka. Paleolithic soils of different ages and their modern analogues were investigated. The age of the buried horizon of volcanic stratified ochre soil was 2500 years old and the horizon of volcanic humus-ochre soil was approximately 11,300 years old. It was found that the biomass of metabolically active prokaryotes of volcanic layered-ochre soil reached 54 μg/g in the control sample and 69 μg/g of the sample in the variant with the addition of chitin, which is approximately 40% of all detected prokaryote biomass. In paleovolcanic soil, the biomass indices of

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Some features of the functional prokaryotic complex of the studied soils were revealed. It was found that the potential activity of the microbial hydrolytic community as measured by the relative response to substrate application increases in the series from modern to paleovolcanic soils. The presence of a number of key genes of the nitrogen cycle (*nifH, amoA, nirK*), as well as the presence of genes responsible for the utilization of n-alkanes, was shown in both modern and buried horizons of the studied soils.

In modern soils, the presence of genes responsible for the possibility of fixing molecular nitrogen from the air was higher compared to those buried. However, the response of the microbial community to the introduction of the resource was higher in buried soils compared to modern ones.

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